Reprogramming CD19-Specific T Cells with IL-21 Signaling Can Improve Adoptive Immunotherapy of B-Lineage Malignancies

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Abstract
Improving the therapeutic efficacy of T cells expressing a chimeric antigen receptor (CAR) represents an important goal in efforts to control B-cell malignancies. Recently an intrinsic strategy has been developed to modify the CAR itself to improve T-cell signaling. Here we report a second extrinsic approach based on altering the culture milieu to numerically expand CAR⁺ T cells with a desired phenotype, for the addition of interleukin (IL)-21 to tissue culture improves CAR-dependent T-cell effector functions. We used electrotreatment of Sleeping Beauty system to introduce a CAR transposon and selectively propagate CAR⁺ T cells on CD19⁺ artificial antigen-presenting cells (aAPC). When IL-21 was present, there was preferential numeric expansion of CD19-specific T cells which lysed and produced IFN-γ in response to CD19. Populations of these numerically expanded CAR⁺ T cells displayed an early memory surface phenotype characterized as CD62L⁺CD28⁺ and a transcriptional profile of naïve T cells. In contrast, T cells propagated with only exogenous IL-2 tended to result in an overgrowth of CD19-specific CD4⁺ T cells. Furthermore, adoptive transfer of CAR⁺ T cells cultured with IL-21 exhibited improved control of CD19⁺ B-cell malignancy in mice. To provide coordinated signaling to propagate CAR⁺ T cells, we developed a novel mutein of IL-21 bound to the cell surface of aAPC that replaced the need for soluble IL-21. Our findings show that IL-21 can provide an extrinsic reprogramming signal to generate desired CAR⁺ T cells for effective immunotherapy. Cancer Res; 71(10); 1–12. ©2011 AACR.

Introduction
Adoptive transfer of antigen-specific T cells has been used to treat and prevent malignancies and opportunistic infections. To overcome immune tolerance to human tumor-associated antigens (TAA), investigators have redirected specificity through the introduction of immunoreceptors. An initial clinical trial showed the safety and feasibility of redirecting T cell specificity to CD19, a TAA expressed on B-cell malignancies (1–3). These clinical data demonstrated that infused T cells were short lived due, in part, to the use of a first-generation chimeric antigen receptor (CAR) that recognized CD19 independent of MHC via chimeric CD3-ζ (signal 1). In response, we developed a second-generation CD19-specific CAR to activate T cells through both CD3-ζ and CD28 endodomains (signals 1 and 2, respectively) to improve T-cell activation (4). To translate this CAR to clinical trials, we established a platform for nonviral gene transfer using the Sleeping Beauty (SB) system and subsequent selective expansion of CAR⁺ T cells recursively cocultured upon CD19⁺ artificial antigen-presenting cells (aAPC) modified from K562 to express CD19 and desired costimulatory molecules (5–7).

In addition to modifying the CAR itself to augment therapeutic potential, we have now manipulated the tissue culture environment to alter the types of CAR⁺ T cells that can be generated. We investigated whether cytokines could be added to cultures to provide a “signal 3” to improve the CAR⁺ T cells response to B-cell malignancies.

One attractive cytokine to use in the culturing of T cells is interleukin (IL)-21, which like IL-2, signals through the cytokine receptor common γ chain (IL-2Rγ). This was selected to be tested on the basis of the published work showing that this cytokine increases tumor-specific T cells (8) and/or natural killer (NK) cells (9, 10) leading to antitumor immunity in animal models. Further, IL-21 provides a T-cell survival signal and can act in conjunction with CD28 to support proliferation and acquisition of effector functions (11). T cells genetically modified to have enforced secretion of IL-21 exhibited improved antitumor effect compared to T cells not modified...
to secrete cytokines (12). Recombinant soluble IL-21 has been intravenously administered in patients with metastatic renal cell carcinoma, melanoma, and lymphoma, and antitumor activity has been observed (13). In contrast to IL-2, IL-21 also inhibits generation of human regulatory T cells in vitro (14).

We hypothesized that altering the culture environment by the addition of IL-21 will lead to improved numeric expansion and functionality of CD19-specific CAR+ T cells. When IL-21 was present with or on aAPC, we found there was a preferential numeric expansion of CAR+ T cells with a preference to propagate subpopulations of (i) CD8+ T cells, (ii) memory T cells, and (iii) naïve T cells, which lysed and produced IFN-γ in response to CD19. This resulted in improved control of CD19+ tumor in a mouse model of human T-cell immunotherapy.

Materials and Methods

Plasmids

The SB transposon CoOpCD19RCD28/pBSBO expresses the human codon optimized (CoOp) second generation CoOpCD19RCD28 CAR under human elongation factor 1-α (hEF-1α) promoter, flanked by the SB inverted repeats (6). To generate membrane-bound IL-21 (mIL-21), the GM-CSF (granulocyte macrophage-colony-stimulating factor) signal peptide sequence was directly fused to the coding sequence of mature human IL-21 which was attached via a modified [amino acid (aa) 108. Ser → Pro] 12 aIgG4 hinge region (aa 99–110), to the 5′ end of a human immunoglobulin γ-4 chain Cγ2 and Cγ3 regions (aa 111–327, UniProtKB P01861), that was fused in frame to human CD4 transmembrane domain (aa 397–418, UniProtKB P01730). After validating the sequence, the human codon optimized cDNA (GENEART) was cloned as a transposon into a SB expression plasmid, pT-MNDU3-eGFP (5) replacing the eGFP sequence to obtain CoOpIL-21-Fc/pT-MNDU3 (Fig. 6A). The SB transposase, SB11 is expressed in cis from the plasmid pCMV-SB11 (6).

Cell lines and their propagation

Daudi coexpressing β3/β2-microglobulin (15; Daudiβ3β2m, a kind gift from Dr Brian Rabinovich, University of Texas MD Anderson Cancer Center (MDACC)), NALM-6 [pre-B cell; ATCC (American Type Culture Collection)], U251T (glioblastoma; a kind gift from Dr Walder Debinski, Wake Forest University, NC), CD19+U251T [ref. 6; expressing truncated CD19, ref. 16] were cultured as described previously (5, 6). NALM-6 cells were transduced using a murine stem cell virus–based retroviral vector encoding enhanced firefly luciferase (eFlLuc; ref. 17) fused with enhanced green fluorescent protein (EGFP; a kind gift from Dr Brian Rabinovich, MDACC). Retrovirus was packaged as previously described (17), concentrated 50× using Amicon Ultra-15 100,000 NMWL centrifugal concentration units (Millipore), mixed with NALM-6 cells in the presence of 8 μg/mL polybren (Sigma) and spinfected for 90 minutes at 2200 RPM/30°C. One week later, EGFP+ NALM-6 cells were sorted on a FACSAria (BD Biosciences). Selective in vitro expansion of genetically modified T cells was carried out using K562-derived aAPC (clone no. 4) coexpressing CD19, CD64, CD86, CD137L, and a membrane-bound IL-15 (mIL-15; coexpressed with EGFP; ref. 15). Clone no. 4 was further modified to express mIL-21 using the construct CoOpIL-21-Fc/pT-MNDU3. Briefly, 106 aAPC were resuspended in 100 μL Amaxa Cell Line Nucleofector Solution V (catalogue no. VPA-1003) along with transposon (CoOpIL-21-Fc/pT-MNDU3, 2 μg) and SB transposase (pCMV-SB11, 2 μg) DNA supercoiled plasmids, transferred to a cuvette and electroporated (Program T-16) using Nucleofector II (Lonza). The transfectants were cultured for a week in complete media and a clone (D2) was obtained by plating at limited dilution after FACS (fluorescence-activated cell sorter) sorting. All cell lines were verified by morphology and/or flow cytometry, tested for Mycoplasma, and conserved in research cell bank on reception.

Generation of CAR+ T cells

CD19-specific CAR+ T cells were generated from peripheral blood mononuclear cells (PBMC) using SB transposition as previously described and depicted in Figure 1 (5). Briefly, 107 to 2 × 107 mononuclear cells, isolated from blood by Ficoll-Paque density gradient centrifugation (GE Healthcare Biosciences AB), were resuspended in 100 μL of Amaxa Nucleofector solution (Human T cell Kit, catalogue no. VPA-1002), along with CAR transposon (CD19RCD28/pBSBO, 15 μg) and SB transposase (pCMV-SB11, 5 μg) DNA plasmids, transferred to a single cuvette and electroporated (Program U-14) on day 0 using Nucleofector II. The cells were rested for 2 to 3 hours at 37°C in incomplete phenol-free RPMI (HyClone) and subsequently cultured overnight in phenol-free RPMI containing 10% FBS and stimulated the next day (day 1) with γ-irradiated (100 Gy) K562-aAPC at a 1:2 T cell/aAPC ratio. Additional γ-irradiated aAPC clone no. 4 were added every 7 days at the same ratio. When used, soluble recombinant human IL-21 (catalogue no. 34-8219-85, eBioscience) was added at a concentration of 30 ng/mL beginning the day after electroporation, and soluble recombinant human IL-2 (IL-2; Chiron) was added to the cultures at 50 U/mL beginning 7 days after electroporation. For experiments where CAR+ T cells were cocultured on mIL-21 K562-aAPC (T cell/aAPC clone D2 ratio 1:2), IL-2 (50 U/mL) was added to the cultures on day 7 after electroporation. All exogenous cytokines continued to be supplemented on a Monday-Wednesday-Friday schedule for 7-day stimulation cycles marked by the addition of aAPC. The cultures were monitored by flow cytometry for the unwanted presence of a CD3+CD56+ cell population and if the percentage exceeded approximately 10% of the total population, which usually occurred between 10 and 14 days of initial coculture with aAPC, a depletion for (CD3+CD56+) NK cells was carried out using CD56 beads (catalogue no. 130-050-401, Miltenyi Biotech Inc.) on LS column (catalogue no. 130-042-401, Miltenyi Biotech Inc.) according to the manufacturer’s instructions. T cells were enumerated every 7 days and viable cells counted based on Trypan blue exclusion using Cellometer automated cell counter (Auto T4 Cell Counter, Nexcelom Bioscience). At the time of electroporation and during the course of coculture, programs “PBMC_human_frozen” and “activated T cell,” respectively, were used for counting cells on the Cellometer. The fold expansion (as compared to day 1) of total, CD3+, and CAR+ cells at the end of 7, 14, 28 days of...
coclulture for individual experiments was calculated and the average compared between culture conditions using a Student’s t test.

Flow cytometry

Up to $10^6$ cells in 100 µL volume were stained with fluorochrome-conjugated [fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein conjugated to cyanine dye (PerCPCy5.5), allophycocyanin (APC)] reagents which unless otherwise stated, were obtained from BD Biosciences; anti–CD3PerCPCy5.5 (catalogue no. 349201, 5 µL), anti–CD3 PerCPCy5.5 (catalogue no. 340949, 4 µL), anti–CD4 APC (catalogue no. 555349, 2.5 µL), anti–CD8 PerCPCy5.5 (catalogue no. 341051, 4 µL), anti–CD19 PE (catalogue no. 555488, 5 µL), anti–CD28 PerCPCy5.5 (catalogue no. 337181, 4 µL), anti–CD64 PE (catalogue no. 558592, 2.5 µL), anti–IL7R PE (catalogue no. 559446, 2.5 µL), anti–mIL-15, mIL-21 PE (catalogue no. 12 7219-73, 2.5 µL), anti–mIL-21 PE (catalogue no. 558592, 2.5 µL), anti–CD137L PE (catalogue no. 559446, 2.5 µL), anti–NKG2D PE (catalogue no. 557940, 2.5 µL), anti–Granzyme B Alexa Fluor 647 (catalogue no. 558598, 10 µL), anti–PD1 APC (catalogue no. 558694, 2.5 µL), anti–PD1 PE (catalogue no. 557924, 2.5 µL), anti–CD4 APC (catalogue no. 557924, 2.5 µL), anti–IFN-γ APC (catalogue no. 555555, 2.5 µL), anti–CD19-specific CAR (catalogue no. 555555, 2.5 µL), anti–CD62L APC (catalogue no. 555555, 2.5 µL), anti–CXCR4 PE (catalogue no. 556437, 3 µL), anti–Perforin PE (Reagent Set no. 556437, 3 µL), anti–HILA (human leukocyte antigen)-ABC APC (catalogue no. 555555, 2.5 µL), anti–CD86 PE (catalogue no. 556588, 2.5 µL), anti–CD64 PE (catalogue no. 558592, 2.5 µL), anti–CD137L PE (catalogue no. 559446, 2.5 µL), anti–IFN-γ APC (catalogue no. 557924, 2.5 µL), anti–pSTAT3 (pY705) PE (catalogue no. 612569, 20 µL), anti–IL-21 PE (catalogue no. 12 7219-73, 2.5 µL, eBioscience), anti–CCR7 PE (catalogue no. FAB197P, 10 µL, R&D Systems) and anti–CXCR4 PE (catalogue no. FAB173P, 10 µL, R&D Systems), FITC-conjugated (catalogue no. H10101C, 3 µL, Invitrogen) and PE-conjugated (catalogue no. H10104, 2.5 µL, Invitrogen) F(ab’)2 fragment of goat anti-human Fcγ was used to detect cell surface expression of the CD19-specific CAR. Blocking of nonspecific antibody binding was achieved using FACS wash buffer (2% FBS and 0.1% Sodium azide in PBS). Data acquisition was on a FACSCalibur (BD Biosciences) using CellQuest version 3.3 (BD Biosciences). Analyses and calculation of median fluorescence intensity (MFI) was undertaken using FCS Express version 3.00.007 (Thornhill).

Chromium release assay

The cytolytic activity of T cells was determined in a standard 4-hour chromium release assay (CRA) as described previously (5, 18).

Intracellular IFN-γ production

$10^6$ T cells were incubated with $0.5 \times 10^6$ stimulator cells in 200 µL culture media along with protein transport inhibitor (BD Golgi Plug containing Brefeldin A, catalogue no. 555028) in a round-bottom, 96-well plate. Following a 4 to 6 hour incubation at 37°C, the cells were stained for expression of CAR at 4°C for 30 minutes. After washing, the cells were fixed, permeabilized (for 20 minutes at 4°C with 100 µL of Cytofix/Cytoperm Buffer, catalogue no. 555028) and stained with APC-conjugated monoclonal antibody (mAb) specific for IFN-γ. Cells were further washed and analyzed by FACSCalibur. T cells treated with a leukocyte activation cocktail [PMA (phorbol 12 myristate 13 acetate) and ionomycin, catalogue no. 550583, BD Biosciences] were used as a positive control.

Induction of STAT3 phosphorylation

$10^6$ CAR+ T cells were incubated with and without aAPC for 30 minutes at 37°C in a V-bottom 96-well plate. The cells were then fixed with 20 excess volumes of 37°C pre-warmed 1 × PhosFlow Lyse/Fix Buffer (catalogue no. 558049, BD
Biosciences) diluted in water at 37°C for 10 minutes to prevent dephosphorylation. Thereafter, pelleted cells were permeabilized by adding BD PhosFlow Perm Buffer III (catalogue no. 55850, BD Biosciences) for 20 minutes on ice, followed by washing with BD Pharmingen Staining Buffer (catalogue no. 5546561). Resuspended cells were then stained with antibodies for pSTAT3, CD3, and CAR for 20 minutes in the dark, washed once with BD Pharmingen Staining Buffer, and resuspended in the same buffer for flow cytometry analysis.

**nCounter analysis digital gene expression system**

Genetically modified T cells (10,000) were lysed in RNeasy lysis buffer (RLT; 5 μL, Qiagen) and frozen for single-use aliquots at −80°C. Lysates were thawed and the selected mRNA content analyzed using the nCounter Analysis System (model no. NCT-SYST-120, NanoString Technologies; ref. 19) after hybridization with a designer reporter code set and capture probe set for 12 hours at 65°C. The probes were designed, synthesized, and hybridized using the nCounter Gene Expression Assay Kit. The posthybridization processing was undertaken using the nCounter Prep Station. Nanominer software was used to carry out normalization compared to internal control and basic statistical analysis on the data. The normalized results are expressed as the relative mRNA level.

**In vivo efficacy of CAR⁺ T cells**

On day 0, 7-week-old NOD.Cg-PrkdcscidIl2rg−/−tm1JSl/J (NSG) mice were intravenously (i.v.) injected via a tail vein with 10⁵ EGFP⁺ effLuc⁺ NALM-6 cells. Mice (n = 5/group) in the two treatment cohorts received via tail vein injection (on days 1 and 9) 2 × 10⁵ of CAR⁺ T cells grown in the presence of IL-2 or CAR⁺ T cells grown in the presence of IL-2 and IL-21. One group of mice (n = 5) bearing tumor received no T cells. Anesthetized mice underwent bioluminescent imaging (BLI) in an anterior–posterior position using a Xenogen IVIS 100 series system (Caliper Life Sciences) 10 minutes after subcutaneous injection (at neck and shoulder) of 150 μL (200 μg/mouse) freshly thawed aqueous solution of α-Luciferin potassium salt (Caliper Life Sciences) as previously described (20). Photons emitted from NALM-6 xenografts were serially quantified using the Living Image 2.50.1 (Caliper Life Sciences) program. At the end of the experiment, mice were euthanized and tissues harvested. Bone marrow was flushed from the femurs using 30Gx1/2 inch needles (BD, catalogue no. 305106) with 2% FBS in PBS. Spleens were disrupted using a syringe in 2% FBS/PBS and passed through a 40 μm nylon cell strainer (BD, catalogue no. 352340) to obtain single cell suspension. Red blood cells from bone marrow, spleen, and peripheral blood were lysed using ACK lysis buffer (Gibco-Invitrogen, A10492) and remaining cells stained for presence of tumor (CD19 and EGFP) by flow cytometry. Statistical analysis of photon flux and tumor burden was accomplished using Student’s t test.

**Results**

**Propagation of CAR⁺ T cells with IL-21**

A master cell-bank of clinical-grade K362-derived aAPC, designated clone no. 4, has been generated through the Production Assistance for Cellular Therapies (PACT) to propagate CD19-specific CAR⁺ T cells to clinically sufficient numbers. These γ-irradiated aAPC selectively propagate CAR⁺ T cells after the electrophoresis of SB plasmids coding for CD19RD28 CAR (5). To investigate extrinsic factors that might improve the therapeutic potential of CAR⁺ T cells, a role for IL-21 was examined in the culturing process on aAPC. We added this soluble cytokine, in addition to soluble IL-2 and mIL-15 on aAPC, and showed selective expansion of CAR⁺ T cells (Fig. 2A). This resulted in a greater number of CD3⁺ and CAR⁺ T cells at 28 days of coculture on clone no. 4 (P < 0.05) compared with T cells receiving IL-2 alone, with differences between the 2 groups already apparent within 2 weeks after electroporation (Fig. 2B and C). Indeed, the average fold expansion of CAR⁺ T cells at 7 (2.75 ± 1.1 fold, n = 7) and 14 (39.2 ± 36.2 fold, n = 7) days after electroporation were significantly higher (P < 0.05) compared to T cells that only received IL-2 (day 7, 0.29 ± 0.3 fold, n = 4; day 14, 0.49 ± 0.36 fold, n = 4). After 4 weeks of coculture on aAPC, the group that received IL-21 had an average 19,800 ± 11,313 (n = 7) fold expansion of CD3⁺ T cells and there was a 35,800 ± 23,285 (n = 7) fold expansion of CAR⁺ T cells. In contrast, T cells that only received IL-2 had an average CD3⁺ fold expansion of 2,280 ± 4,227 (n = 4) and CAR⁺ T cells expanded by an average of 2,680 ± 4,919 (n = 4) fold. The average total cell numbers at 7 (4.1 × 10⁷ vs. 4.7 × 10⁶) and 14 (3.1 × 10⁶ vs. 2.7 × 10⁵) days after electroporation were significantly higher (P < 0.05) in cultures receiving IL-21, as compared to cultures receiving only IL-2, which was due to an increased average number of average CD3⁺ T cells (day 7, 4.1 × 10⁷ vs. 3.0 × 10⁶; day 14, 3.5 × 10⁵ vs. 2.4 × 10⁵; P < 0.05). These data suggest the addition of soluble recombinant IL-21 to the culture environment augments the expansion of CD3⁺ CAR⁺ T cells on aAPC clone no. 4.

**IL-21 results in qualitative differences in CAR⁺ T cells**

CAR⁺ T cells cultured only in the presence of IL-2 produced low amounts of IFN-γ in response to CAR binding CD19 and expressed low levels of granzyme B. Expression of this cytokine and granzyme is associated with improved antitumor activity, therefore we investigated whether the presence of IL-21 could augment expression of these factors as T cells were propagated on aAPC. We measured mRNA levels using the nCounter Analysis System and IFN-γ and Granzyme B were found to be significantly elevated in populations of T cells receiving IL-21 (Fig. 3A). At day 28 of culture, we observed a 3-fold increase in IFN-γ (313 vs. 100) and 40-fold increase in granzyme B (4,458 vs. 110) mRNA transcript levels in T cells grown in IL-2 and IL-21, as compared to those grown in soluble IL-2. We demonstrated a significant increase in granzyme B protein levels (mean 89.5% vs. 17%) and CD19-dependent IFN-γ production (mean 55% vs. 0.1%) in the CAR⁺ T cells receiving IL-21, as compared to those grown in soluble IL-2 (Fig. 3B). Thus, the mRNA measurements are consistent with the phenotype data showing an increase in expression of IFN-γ and granzyme B after coculture of CAR⁺ T cells with IL-2 and IL-21. We have previously generated CD19RD28 CAR⁺ T cells on CD19⁺ aAPC in the presence of soluble IL-2 which tended
to result in a preferential outgrowth of CAR\(^+\) T cells with a predominance of CD4\(^+\) T cells (5). Recognizing that CD8\(^+\) T cells contribute to antitumor immunity, we sought a method to improve the outgrowth of CD8\(^+\) CAR\(^+\) T cells on aAPC. IL-21 has been shown to help propagate CD8\(^+\) effector T cells (21), therefore the genetically modified T cells were cocultured with aAPC in the presence of exogenous IL-21 and IL-2 for 28 days which resulted in a trend toward improved outgrowth of CD8\(^+\) CAR\(^+\) compared with CD4\(^+\) CAR\(^+\) T cells.

Day 28 was selected as an endpoint for tissue culture and subsequent analyses since over this time period CAR\(^+\) T cells expand to numbers sufficient for clinical translation (Fig. 2). The T cells receiving IL-21 had 1.8-fold higher number of average CD8\(^+\) T cells (24.2/25.3) compared to the T cells receiving just IL-2 (13.4/10.9) at day 28 of coculture (Fig. 3D). At the time of analysis, there was increased CAR expression on the T cells exposed to IL-21 compared to T cells cultured with only IL-2 (mean 90%/7.5 vs. 66%/7; \(P < 0.01\)) which indicates that manipulating the cytokine milieu can improve the outgrowth of T cells with increased expression of CAR (Fig. 3C). In aggregate, the addition of IL-21 compared to culturing only with IL-2, results in T cells containing subpopulations that have improved effector function, a trend toward CD8\(^+\) phenotype, and augmented CAR expression.

**IL-21 results in propagation of subpopulations of CAR\(^+\) T cells with memory and naïve phenotypes**

T cells propagated over a prolonged time in tissue culture tend to mature to a differentiated phenotype which may compromise their therapeutic potential. To determine if IL-21 can curtail this differentiation of genetically modified T cells, we examined the expression of (i) eomesodermin (EOMES) which controls cytolytic development and function of T cells, and has recently been shown to be reduced in naïve-derived effector cells (22-24) and (ii) killer cell lectin-like receptor subfamily G, member 1 (KLRG1), an inhibitory receptor that is expressed by senescent T cells (25, 26). At day 28, the T cells cultured only with IL-2 had increased levels of mRNA species coding for EOMES and KLRG1 showing that this cytokine promoted differentiation of CAR\(^+\) T cells on aAPC. However, when IL-21 was added there was a 7-fold and 54-fold reduction in EOMES and KLRG1, respectively (Fig. 3A). To expand on the mRNA data, we examined the cell surface expression for flow}

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**Figure 2.** Propagation of genetically modified T cells on aAPC. A, T-cell expression of CAR before and after propagation on aAPC. The rate of T-cell growth cultured on \(\gamma\)-irradiated aAPC for 28 days in the presence of IL-2 (\(n = 4\)) or IL-2 and IL-21 (\(n = 7\)). The total number of B, CD3\(^+\) cells, and C, CAR\(^+\) T cells propagated over time. Small upward arrows indicate the addition of \(\gamma\)-irradiated aAPC to the culture. Inferred cell counts were calculated assuming all viable cells were carried forward through each stimulation cycle. Mean ± SD is shown; *, \(P < 0.05\); **, \(P < 0.001\).
cytometry markers of naïve, memory, and differentiated T cells. A naïve phenotype was defined by the presence of CD62L and CD45RA, whereas a central memory (T_{CM}) phenotype can be defined by the expression of CD28, CD62L, CCR7, and CD45RO on T cells (27–29). Differentiated effector cells typically lose expression of these markers upon prolonged culturing, and exhausted cells upregulate expression of PD-1 and PD-L1 (30). CAR\textsuperscript{+} T cells grown in the presence of IL-2 and IL-21 exhibited markers consistent with both naïve and memory cells and lacked expression of PD-1/PDL-1 (Fig. 4A). In addition, the lack of (2%) CD57 expression supports absence of exhaustion among the CAR\textsuperscript{+} T cells (31). At day 28 of culture, the CAR\textsuperscript{+} T cells expressed CD28 (mean 69%, range: 22%–88%), CCR7 (mean 16%, range: 0.9%–60.5%), CD62L (mean 50%, range: 22%–75%), CD45RO (93%, range: 84%–98%), and IL7R\textalpha (mean 26%, range: 9%–37%). Among the CAR\textsuperscript{+} T cells were T cells with a T_{CM} phenotype exemplified by the coexpression of CD28\textsuperscript{+}CD62L\textsuperscript{−} (34%, range: 11%–59%), CD28\textsuperscript{−}CCR7\textsuperscript{+} (mean 18%, range: 0.8%–56.5%), CD62L\textsuperscript{−}.CCR7\textsuperscript{+} (10%, range: 0.5%–48%), CD45RO\textsuperscript{−}CD45RA\textsuperscript{+} (mean 19%, range: 2%–63%). In summary, the addition of IL-21 supports the numeric expansion of CAR\textsuperscript{+} T cells on aAPC that contain memory and naïve subpopulations.

**Redirected specificity of CAR\textsuperscript{+} T cells numerically expanded with IL-21**

T cells expressing CAR and propagated in the presence of IL-2 and IL-21 were able to specifically lyse CD19\textsuperscript{+} tumor targets (Fig. 4B). At an effector:target ratio of 5:1, the CAR\textsuperscript{+} T cells could lyse an average 51% (range: 32%–66%) of CD19\textsuperscript{+} Daudi\textsuperscript{b2m} cells and an average 38% (range: 25%–60%) of CD19\textsuperscript{+} U251T (glioma) targets as compared to parental CD19\textsuperscript{−} U251T (Fig. 4A). At day 28 of culture, the CAR\textsuperscript{+} T cells expressed CD28 (mean 69%, range: 22%–88%), CCR7 (mean 16%, range: 0.9%–60.5%), CD62L (mean 50%, range: 22%–75%), CD45RO (93%, range: 84%–98%), and IL7R\textalpha (mean 26%, range: 9%–37%). Among the CAR\textsuperscript{+} T cells were T cells with a T_{CM} phenotype exemplified by the coexpression of CD28\textsuperscript{+}CD62L\textsuperscript{−} (34%, range: 11%–59%), CD28\textsuperscript{−}CCR7\textsuperscript{+} (mean 18%, range: 0.8%–56.5%), CD62L\textsuperscript{−}.CCR7\textsuperscript{+} (10%, range: 0.5%–48%), CD45RO\textsuperscript{−}CD45RA\textsuperscript{+} (mean 19%, range: 2%–63%). In summary, the addition of IL-21 supports the numeric expansion of CAR\textsuperscript{+} T cells on aAPC that contain memory and naïve subpopulations.

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of IL-2 and IL-21 were more effective in controlling tumor growth as compared to CAR+ T cells grown in the presence of IL-2 (day 8, P < 0.01; day 13, P < 0.05; day 17, P < 0.05) and as compared to mice that did not receive T cells (days 8, 13, 17, P < 0.01; Fig. 5A and B). Groups of mice receiving no T cells or CAR+ T cells grown in the presence of IL-2 showed similar rates of tumor growth (day 8, P = 0.11; day 13, P = 0.2; day 17, P = 0.07). Tissues from mice were assessed for EGFP+ CD19+ NALM-6 and consistent with the BLI data, we observed that the tumor burden was significantly reduced in mice receiving CAR+ T cells grown in the presence of IL-2 and IL-21, as compared to mice receiving no T cells or CAR+ T cells grown in the presence of IL-2 (Fig. 5C and D). The presence of NALM-6 blasts in peripheral blood was lower in the IL-2 and IL-21 (1.8 ± 0.92, mean ± SD) group as compared to mice that received CAR+ T cells cultured with IL-2 (20.4 ± 20.3, P < 0.05) or no T cells (3.8 ± 0.6, P < 0.01). There was also a significant reduction in average tumor burden in the bone marrow after administration of T cells that were cultured with IL-2 and IL-21 group (48.5 ± 4.2) as compared to IL-2 group (86.6 ± 7.8, P < 0.00001) or no T-cell group (86.6 ± 7.9, P < 0.001). The difference in average splenic tumor burden was more apparent (P < 0.00001) comparing mice that received CAR+ T cells cultured with IL-2 and IL-21 (30.9 ± 18.8) vs. mice that were infused with CAR+ cells cultured with IL-2 (88.3 ± 4). The tumor burden was similar in the blood (P = 0.07) and bone marrow (P = 0.49) between the mice that received no T cells and mice that received CAR+ T cells cultured with IL-2. Thus, these in vivo data confirm that CD19-specific CAR+ cells propagated on aAPC in presence of IL-2 and IL-21 are superior in controlling tumor growth.

A membrane-bound mutein of IL-21 can replace soluble recombinant IL-21

Signaling through 2nd generation CAR and cytokine receptor(s) occurs in the tissue culture environment enabling aAPC to selectively propagate T cells that receive signals 1, 2, and 3. To improve the coordination between these sig-
Bone Marrow
Spleen

...were harvested and analyzed by flow cytometry for expression of CD19 and EGFP. Representative flow cytometry dot plots for tumor at various sites are shown. C, at the end of the experiment (day 21) mice were euthanized and tissues (blood, bone marrow, spleen) were harvested and analyzed by flow cytometry for expression of CD19 and EGFP. Representative flow cytometry dot plots for tumor at various sites are shown. E, the percentage of CD19*EGFP* cells present in mice from the 3 groups (mean ± SD) is shown along with statistical significance (*, P < 0.05, **, P < 0.01).

Figure 5. Efficacy of CAR* T cells in mice. NSG mice i.v. injected (day 0) with 10^5 EGFP*effLuc* NALM-6 tumor received on days 1 and 9, 2 x 10^7 CAR* T cells grown in the presence of IL-2 or IL-2 & IL-21, or received no T cells. A, false-colored images representing photon flux from NALM-6 tumor only, Tumor & CAR* T cells (IL-2), Tumor & CAR* T cells (IL-2 & IL-21). B, Tumor & CAR* T cells (IL-2) (n=5) Tumor & CAR* T cells (IL-2 & IL-21) (n=5) Tumor only. Days 0 5 10 15 20

...were propagated on PBMC electroporated with SB system to express CD19R-STAT3. CAR* T cells, aAPC (clone no. D2), were stimulated for 30 minutes on aAPC showed specific lysis of CD19* target cells and a 13-fold increase in IFN-γ production when cells were stimulated with CD19* U251T targets over control CD19 ne−/C6 targets (Fig. 7D). To examine if mIL-21 can directly activate T cells, we evaluated the phosphorylation status of STAT3. CAR* T cells that were propagated for 28 days with IL-2 plus mIL-21neg aAPC (clone no. 4) and IL-2 plus mIL-21 aAPC (clone no. D2), were stimulated for 30 minutes on aAPC with (clone no. D2) or without mIL-21 (clone no. 4). T cells cultured with IL-2 and soluble IL-21 stimulated with aAPC (clone no. 4) along with soluble IL-21 were used as positive controls. CAR* T cells cultured on mIL-21+ aAPC in...
the presence of IL-2 when stimulated with mIL-21þ aAPC resulted in an increase in MFI of pSTAT3 (27.1), over the unstimulated control T cells (11.6), or when stimulated with aAPC lacking mIL-21 (13.2; Fig. 7C). When CARþ T cells which had been propagated on aAPC (clone no. 4) in the presence of IL-2 (and had not seen IL-21 in any form) were exposed to mIL-21þ aAPC, there was a similar increase in MFI of pSTAT3 (25.2) compared to the controls (unstimulated, 11.1; aAPC lacking mIL-21, 14.3). These data show that mIL-21 on aAPC is functional and capable of activating T cells through the STAT3 pathway. In aggregate, these data suggest that mIL-21þ aAPC can replace soluble recombinant IL-21 to selectively propagate CARþ T cells with redirected specificity.

Discussion

The tissue culture environment can be modified to play a critical role in the growth and function of T cells propagated in vitro. In the current study, we show that the addition of IL-21 to the culture milieu in soluble form or presented in conjunction with antigen on aAPC results in improved effector function and preservation of naïve/memory phenotype of CARþ T cells which predicts for improved therapeutic effect in clinical trials.
Initial (first generation) CARs commonly link a scFv to a single signaling moiety (e.g., CD3-ζ) and these typically showed a lack of sustained persistence in vivo (2). Therefore, the CAR design was modified to add one or more costimulatory signaling domains of CD28 (4), 4-1BB (32, 33), OX40 (34) to generate “second” or “third” generation CARs. We have previously described our second generation CAR, CD19RCD28, which provides CD19-dependent signaling through CD3ζ and CD28 resulting in improved persistence and antitumor effect (4). Operating under the premise that T cells propagated in a CAR-dependent manner ex vivo may select for T cells with sustained proliferation in vivo, we developed a culturing system based on aAPC to selectively numerically expand CAR⁺ T cells. Such aAPC derived from K562 can be tailored to express cell-surface antigen (35) or intracellular antigen presented by restricting MHC (36, 37) in the context of desired and introduced T-cell costimulatory molecules (37–40). Our aAPC (clone no. 4) were also engineered to express mIL-15 and this membrane-bound cytokine is present throughout our culturing process in addition to the addition of soluble cytokines. The addition of IL-21-mediated signaling during the culturing process to selectively propagate T cells expressing a second generation CD19-specific CAR resulted in an outgrowth of T cells on aAPC with a desired phenotype and improved function when assessed in vitro and in vivo. Thus, from the perspective of manufacturing CAR⁺ T cells for clinical application it appears beneficial to include IL-21 in the culturing process.

It has been previously shown that the tissue culture microenvironment can be altered with cytokines for improved T-cell function. For example, T cells can be primed using cytokines to augment immune responses after adoptive transfer. In some cases, this is dependent on generation of memory phenotype which requires the presence of IL-15 and IL-21 (41, 42). This memory phenotype predicts for improved persistence as shown for T cells cultured with IL-7, IL-15, and IL-21, compared to T cells propagated with IL-2 (8, 43, 44). Our data provide supportive evidence that not only can the CAR be manipulated, but the choice of additional cytokines influences the number and character of the propagated CAR⁺ T cells.

Membrane-bound cytokines (45, 46) offer an attractive approach for delivering a desired cytokine to the immediate microenvironment of the T cell-aAPC synapse along with alleviating the need to add the soluble recombinant (expensive) cytokine to the culture system and avoiding the need to procure clinical-grade cytokine for clinical applications. Membrane-bound IL-15 has been used to propagate
T cells (6, 47) and NK cells (48, 49) on aAPC derived from K562 cells. Our aAPC (clone no. 4) used in this study express mIL-15 and is being used in our clinical trials infusing patient- and donor-derived CD19-specific CAR T cells after autologous and allogeneic hematopoietic stem-cell transplantation. Building upon the success of aAPC expressing mIL-15, we further modified the aAPC clone no. 4 to coexpress mL-21 and suggest that this genetic approach to aAPC design may avoid the need to add soluble recombinant IL-21 to the culture.

Our data show that the SB system and aAPC platform can be manipulated to culture T cells to receive 3 coordinated signals. This was achieved by influencing intrinsic (CAR) and now extrinsic (tissue culture) factors to improve the therapeutic potential of genetically modified and propagated T cells. Activation through second generation CAR, triggered by CD3ζ (signal 1) and CD28 (signal 2), and common γc receptor (triggered by IL-21, signal 3) results in generation of CAR T cells that have improved ability to respond to CD19 compared to T cells cultured without IL-21. The ability to augment signal 3 leads to an outgrowth of CAR T cells on aAPC that have desired properties for use in clinical trials.

### References

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Reprogramming CD19-Specific T Cells with IL-21 Signaling Can Improve Adoptive Immunotherapy of B-Lineage Malignancies

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