Combining CD19 Redirection and Alloanergization to Generate Tumor-Specific Human T Cells for Allogeneic Cell Therapy of B-Cell Malignancies

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Abstract

Allogeneic hematopoietic stem-cell transplantation can cure some patients with high-risk B-cell malignancies, but disease relapse following transplantation remains a significant problem. One approach that could be used to augment the donor T-cell–mediated antitumor effect is the infusion of allogeneic donor–derived T cells expressing a chimeric antibody receptor (CAR) specific to the B-cell antigen CD19. However, the use of such cells might result in toxicity in the form of graft-versus-host disease mediated by CD19-specific (CD19-CAR) T cells possessing alloreactive endogenous T-cell receptors. We therefore investigated whether nonalloreactive tumor-specific human T cells could be generated from peripheral blood mononuclear cells of healthy donors by the combination of CD19 redirection via CAR expression and subsequent alloanergization by allosimulation and concomitant blockade of CD28-mediated costimulation. Alloanergization of CD19-CAR T cells resulted in efficient and selective reduction of allosponses in both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, including allospecific proliferation and cytokine secretion. Importantly, T-cell effector functions including CAR-dependent proliferation and specific target cytolysis and cytokine production were retained after alloanergization. Our data support the application of CD19 redirection and subsequent alloanergization to generate allogeneic donor T cells for clinical use possessing increased antitumor activity but limited capacity to mediate graft-versus-host disease. Immunotherapy with such cells could potentially reduce disease relapse after allogeneic transplantation without increasing toxicity, thereby improving the outcome of patients undergoing allogeneic transplantation for high-risk B-cell malignancies. Cancer Res; 70(10); OF1-10. ©2010 AACR.

Introduction

Disease recurrence is a major cause of mortality after allogeneic hematopoietic stem-cell transplantation (HSCT) for patients with poor-risk B-lineage malignancies (1–3). Adoptive transfer of allogeneic donor–derived T cells possessing additional antitumor activity has the potential to reduce relapse after allogeneic HSCT. The combination of such an approach with a strategy to selectively control allosponses to limit toxicity from graft-versus-host disease (GvHD) might improve the outcome of allogeneic HSCT for patients with B-cell malignancies.

The introduction of a chimeric antibody receptor (CAR; ref. 4) to redirect human T-cell specificity is one strategy to enhance desired T-cell–mediated antitumor activity (5). CARs typically consist of an HLA-independent high-affinity antigen recognition domain formed from extracellular single-chain immunoglobulin variable fragments, linked to one or more cytoplasmic T-cell activation domains, including CD3-ζ. Infusion of patient-derived T cells expressing a tumor-associated antigen–specific CAR has resulted in some disease responses in early clinical trials for CD20\textsuperscript{+} B-cell lymphomas and GD\textsubscript{2} neuroblastoma, but in other trials, apparently limited in vivo persistence of CAR T cells restricted their therapeutic potential (6–10).

CD19, an early cell-surface B-lineage–restricted molecule, is expressed on both normal B cells and a wide range of human B-cell malignancies (11). Therefore, human CD19-specific CAR T cells have been developed to redirect a T-cell–mediated antitumor effect (12, 13). Second-generation CD19-CAR cells possessing modified costimulatory signaling domains fused to chimeric CD3-ζ have improved in vivo persistence and antitumor efficacy in mice (14, 15). To facilitate the clinical use of CAR T cells, we and others have recently used an augmented nonviral gene insertion strategy [the Sleeping Beauty (SB) transposon/transposase system] to introduce a second-generation CD19-CAR into primary human T cells (16–19).
CAR+ T cells have not yet been infused in the human allogeneic setting. Allogeneic CAR+ T cells could provide an additional donor-derived T-cell–mediated antitumor effect to protect against tumor relapse after allogeneic HSCT. The use of allogeneic rather than patient-derived CAR+ T cells would also eliminate the risk of tumor cell contamination. Additionally, reconstituting donor-derived T cells that have not undergone CD19 redirection but are reactive to recipient hematopoietic tissue–restricted minor histocompatibility antigens could provide protection against CD19+ tumor precursors (20, 21), which would not be selectively targeted by CD19-CAR cells. However, CAR+ T cells possess endogenous αβ T-cell receptors (TCR), and infused allogeneic CAR+ T cells bearing αβ TCR specific to recipient alloantigens could potentially mediate GvHD. Nonselective approaches to reducing alloreactivity after allogeneic HSCT (such as pharmacologic immunosuppression) would likely reduce the ability of allogeneic CAR to expand and function in vivo. Strategies have been developed to selectively reduce alloreactivity in donor T cells after allogeneic HSCT (22–24). We and others have previously shown that one such strategy, alloanergization by allotransplantation concomitant with blockade of CD28-mediated costimulation, effectively and selectively reduces alloreactivity of HLA-mismatched human peripheral blood mononuclear cells (PBMC; refs. 25–27). Furthermore, we have successfully applied this strategy in two prior clinical trials to selectively reduce alloreactivity of HLA-mismatched donor–derived T cells in the setting of haploidentical bone marrow trans plantation (28, 29). A significant proportion of SB-modified CD19-CAR cells propagated on artificial CD19+ antigen-presenting cells (aAPC) bearing the costimulator ligand CD86 express CD28 (18), suggesting that alloanergization would be a suitable technique to selectively reduce alloreactivity in such cells.

To develop a clinical strategy to increase antitumor activity in allogeneic donor T cells while controlling alloreactivity, we investigated whether our established strategy of alloanergization could abrogate alloreponses of second-generation CD19-CAR cells without loss of viability, phenotype, and CAR-dependent T-cell effector functions.

Materials and Methods

Plasmids. The SB transposon contains the codon-optimized (CoOp) second-generation CD19RCD28 CAR, specific for human CD19, flanked by the SB inverted repeats. The ampicillin resistance gene (AmpR) and origin of replication from the plasmid pCoOpCD19RCD28/pT-MNDU3 (18) were replaced with the DNA fragment encoding the kanamycin resistance gene (KanR) and origin of replication (ColEl) from the pEK vector (30), and the human elongation factor-1α (hEF-1α) promoter fragment from pVitro4 vector (InvivoGen) was swapped with MNDU3 promoter to generate CD19RCD28/pSBSO [also referred to as CD19RCD28mZ/(CoOp)/pSBSO]. The SB hyperactive transposase SB11, under the control of cytomegalovirus (CMV) promoter from the plasmid pCMV-SB11 (18), was ligated with the pEK vector fragment encoding KanR and ColEl to generate pKan-CMV-SB11.

Cell lines. CD19+ Daudi (Burkitt lymphoma) and CD19neg K562 (erythroleukemia) cells were obtained from American Type Culture Collection. CD19+ NALM-6 (pre-B cell) and CD19+ Granta 519 (B-cell non–Hodgkin lymphoma) cells were from DSMZ. CD19neg LM7 (osteosarcoma) was a kind gift from Dr. Eugenie Kleinerman (M.D. Anderson Cancer Center, Houston, TX). Cell lines, including aAPC, were maintained in HyQ RPMI 1640 (Hyclone) supplemented with 2 mmol/L Glutamax-1 (Invitrogen) and 10% heat-inactivated FCS (HyClone; 10% RPMI). CD19+ K562 targets were maintained in 10% RPMI with HygroGold (hygromycin B, 0.4 mg/mL; InvivoGen) as described (31). CD19neg U251T (glioblastoma) was a kind gift from Dr. Waldemar Debinski (Wake Forest University, Winston-Salem, NC). U251T were transfected with SB DNA plasmid (pSBSO) expressing truncated CD19 (ΔCD19/pSBSO) to generate CD19neg U251T. The U251T cell lines were maintained in 10% RPMI with G418 (0.2 mg/mL; InvivoGen).

Generation of CD19-CAR cells. PBMC isolated by Ficoll-Paque (GE Healthcare) density gradient centrifugation of peripheral blood obtained from healthy adult volunteer donors after informed consent from Gulf Coast Regional Center (Houston, TX) were cultured in HyQ RPMI 1640 (Hyclone) supplemented with 2 mmol/L Glutamax-1 (Life Technologies-Invitrogen) and 10% heat-inactivated defined FCS (HyClone). The SB transposon/transposase were electrotransferred (Amaxa/Lonza) into T cells derived from PBMC and a population of CD19-CAR cells were numerically expanded on γ-irradiated (100 Gy) K562-artificial antigen-presenting cells (aAPC; ref. 32) expressing CD19, 4-IBBL, CD86, CD64, and membrane-bound interleukin (IL)-15 as previously described (Fig. 1A and B; ref. 18).

Alloanergization of CD19-CAR cells and measurement of secondary alloreponses. Equal numbers of CD19-CAR cells and γ-irradiated (3.5 Gy) first-party allostimulator PBMC (isolated from healthy unrelated adult volunteers after consent on an Institutional Review Board–approved protocol) were cocultured in culture medium (RPMI containing penicillin/streptomycin and 10% human AB serum; Sigma-Aldrich) with or without humanized monoclonal anti-B7.1 (clone h1F1) and anti-B7.2 (h3D1) antibodies (10 μg/106 cells; Wyeth) as described (26) and outlined in Fig. 1C. After 72 hours, cocultures with anti-B7 antibodies (“alloanergized”) and without anti-B7 antibodies (“nonanergized”) were washed and resuspended. Secondary alloreponses were measured after restimulation with γ-irradiated first- or third-party allostimulator PBMC or soluble CD3 and CD28 antibodies (10 μg/mL; Beckman Coulter). Proliferation was determined by thymidine incorporation as described (26). Alloanergization efficiency (AE) was calculated as AE = 100 − [100 × [secondary alloproliferation (alloanergized cells)/secondary alloproliferation (nonanergized cells)]]

Additionally, CD19-CAR cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) before restimulation (26). Allo-specific precursor frequency was calculated as previously described (33), using Flowjo V4 software. Cytokine responses after allorestimulation were also measured using intracellular cytokine flow cytometry (ICC;
ref. 34). Positive controls were stimulated with staphylococcal enterotoxin B (SEB; 10 μg/mL; Sigma-Aldrich). Stimulus-specific responses were calculated by subtracting values for unstimulated cells from values for stimulated cells.

**CD19 depletion of allostimulator PBMC.** Allostimulator PBMC were depleted of CD19+ B cells by labeling with conjugated anti-CD19 paramagnetic microbeads (Miltenyi Biotec, Gladbach, Germany) and passaged through an LD column and midiMACS magnetic device before irradiation. PBMC were analyzed by flow cytometry to assess efficiency of depletion.

**Flow cytometry.** Unless stated, antibodies were from Beckman Coulter. For alloresponses assessment, cells were stained with anti-CD3 (clone UCHT1), anti-CD4 (13B8.2), anti-CD8 (SFCI21Thy2D3), anti-CD14 [M5E2, Becton Dickinson (BD)], and anti-CD19 (RMO52) antibodies conjugated to FITC, phycoerythrin (PE), energy-coupled dye, PE-Cy5, and/or PE-Cy7. Viability was assessed with 7-amino actinomycin D (BD). For ICC, cells were stained for surface molecules, fixed, permeabilized, and then stained with IFN-γ-FITC (4S.B3, BD) and tumor necrosis factor-α (TNF-α)-PE-Cy7 (MAB11, BD). Events were acquired on a Cytomics FC500 flow cytometer and analyzed using Flowjo version 4. For detection of cell-surface expression of CD19-CAR, goat F(ab')2 anti-human IgG (γ)-PE (Invitrogen) or goat F(ab')2 anti-human IgG (γ)-FITC (Jackson ImmunoResearch Laboratories, Inc.) was used (1/20 dilution) with anti-CD4 (RPA-T4, BD), anti-CD8 (SK1, BD), and anti-CD3 (SK7, BD). Anti-CD27 (M-T271), anti-CD28 (L293), anti-CD45RO (UCHL1), anti-CD62L (DREG56), and anti-CD95 (DX2) were used for memory cell pheno-subtyping (all BD). Nonspecific antibody binding was blocked using 2% fetal bovine serum in PBS (with sodium azide).

For CAR-dependent IFN-γ secretion, 10^5 CD19-CAR cells were incubated for 4 to 6 hours with 0.5 × 10^6 stimulator cells or phorbol 12-myristate 13-acetate (5 ng/mL) and ionomycin (500 ng/mL) in 200 μL of culture medium with Golgi Plug (BD), fixed, permeabilized, and stained for intracellular IFN-γ (B27, BD). Events were acquired on a FACSCalibur flow cytometer and analyzed using CellQuest version 3.3 (both from BD).

**Measurement of cytotoxicity.** The cytolytic activity of CD19-CAR cells was determined by a 4-hour chromium release assay (13). CD19-CAR cells were incubated with 5 × 10^3 51Cr-labeled target cells in V-bottomed 96-well plates (Costar). The percentage of specific cytolysis was calculated from the release of 51Cr, as described, using a TopCount NXT (Perkin-Elmer).

**Statistics.** Statistical analysis was done with GraphPad Prism v4. A P value of <0.05 was used to reject the null hypothesis. Data are presented as mean (±SD) unless otherwise stated. P values are for two-tailed paired t tests.

**Results**

**Proliferative alloresponses of CD19-CAR cells were specifically reduced after alloanergization.** We screened CD19-CAR T-cell lines from six adult donors for secondary
(recall) alloproliferative responses after allostimulation (priming) and subsequent allostimulation with γ-irradiated PBMC from 18 different unrelated adult volunteers. Secondary alloproliferative responses were detectable in all CD19-CAR T-cell lines. We next examined the efficacy and specificity of alloanergization in reducing secondary allosponses. Viability of CD19-CAR cells was similar before (87 ± 7%, CD4+ and 92 ± 4%, CD8+) and after alloanergization (83 ± 11%, CD4+ and 90 ± 5%, CD8+). Alloanergized CD19-CAR cells were hyporesponsive to first-party allostimulation. This was not due to a change in kinetics of the alloproliferative response (Fig. 2A). Reduction of peak first-party–specific alloproliferation after alloanergization was seen in all CD19-CAR cell lines, whereas there was no significant change in third-party–specific alloproliferation or mitogen-stimulated proliferation, showing that hyporesponsiveness was specific to alloantigens used during alloanergization (Fig. 2B). The median efficiency of alloanergization was 82% (range, 33–96%) with a median 5.4-fold (range, 1.5–26) reduction in first-party alloproliferative responses. Third-party and mitogen-stimulated proliferation were not reduced (Fig. 2C). These data show that alloanergization specifically reduced alloproliferation of CD19-CAR cells, consistent with our previous data for alloanergization of nongenetically modified human PBMC (26, 35).

The use of CD19-depleted alloanergizers resulted in a further reduction of residual allosponses after alloanergization of CD19-CAR cells. Proliferation of CD19-CAR cells after stimulation with allogeneic PBMC containing CD19+ cells could result either from alloantigen-specific stimulation via endogenous TCR or from direct CD19-mediated stimulation via introduced CAR. The latter could provide a confounding factor in our proliferation assays. Therefore, we sought to determine whether the presence of CD19+ cells within alloanergizer PBMC affected the residual proliferation of alloanergized CD19-CAR cells. We depleted CD19+ cells from alloanergizer PBMC before γ-irradiation, resulting in a median 500-fold reduction in CD19+ cells. Proliferative responses after alloanergization of CD19-depleted alloanergizer PBMC, consistent with retention of alloanergizer capacity. However, the use of CD19-depleted alloanergizer PBMC resulted in a lower residual proliferation after alloanergization of CD19-CAR T cells compared with the use of unsorted alloanergizer PBMC, suggesting that a direct stimulatory effect mediated by CD19+ cells within alloanergizer PBMC contributed to the

Figure 2. Alloproliferation of CD19-CAR cells is specifically reduced by alloanergization. A, mean values for proliferation ([3H]thymidine incorporation) of nonanergized and alloanergized CD19-CAR cells after alloanergization for 18 stimulator-responder pairs; bars, SD. B, peak proliferation in nonanergized and alloanergized CD19-CAR cells after restimulation with first- or third-party alloanergizers or mitogenic CD3 and CD28 antibodies. Symbols, means of triplicate values for unique stimulator-responder pairs; horizontal bars, mean values for all pairs. C, fold reduction in proliferation of alloanergized CD19-CAR cells (compared with nonanergized cells) after alloanergization of CD19-depleted PBMC as alloanergizers. Results depict three separate experiments; horizontal bars and adjacent numbers depict median values.
residual proliferation after alloanergization. As a result, measured efficiency of alloanergization was significantly higher (median, 93%; range, 87–94%) using CD19-depleted allostimulator PBMC when compared with unsorted allostimulator PBMC (median, 72%; range, 55–77%; \( P = 0.04 \); Fig. 2D).

**Alloanergization reduced alloproliferation in both CD4+ and CD8+ CD19-CAR cells.** As the propagated CD19-CAR cells contained both CD4+ and CD8+ T cells, we next determined whether alloanergization reduced alloproliferation in both cellular subsets. We labeled CD19-CAR cells with CFSE before allorerestimulation and measured proliferation by CFSE dilution. After 6 days of allorestitution, 21% \( \pm 6.2\% \) and 15% \( \pm 6.1\% \) of nonanergized CD4+ and CD8+ CD19-CAR cells had proliferated. This represented median CD4+ and CD8+ alloprecursor frequencies of 1.5% (range, 1.0–1.8%) and 1.4% (range, 1.3–1.9%), respectively. In contrast, the mean percentages of CD4+ and CD8+ T cells proliferating after allorestitution of alloanergized CD19-CAR cells were significantly lower at 6.9% \( \pm (4.7\% \) and 4.1% \( \pm (3.6\% \), respectively. Importantly, proliferation of both CD4+ and CD8+ CD19-CAR cells after mitogenic stimulation was unaffected by alloanergization (Table 1). Thus, CD19-CAR cells contain both alloproliferative CD4+ and CD8+ T cells, and alloproliferation in both these subsets was specifically reduced after alloanergization.

**Alloanergization of CD19-CAR cells reduced allospecific cytokine production.** Alloreactive human CD4+ and CD8+ T cells secrete proinflammatory cytokines (predominantly IFN-\( \gamma \) and TNF-\( \alpha \)) after HLA-mismatched allosemination (34). Therefore, we used ICC to examine the effect of alloanergization on allospecific cytokine responses of CD19-CAR cells. Allospecific cytokine+ T cells were detected within CD19-CAR T-cell populations with cell frequencies of 3.8% \( \pm (2.7\% \), CD4+IFN-\( \gamma \), 0.8% \( \pm (0.7\% \), CD8+IFN-\( \gamma \), 2.5% \( \pm (1.4\% \), CD4+TNF-\( \alpha \), and 0.8% \( \pm (0.7\% \), CD8+TNF-\( \alpha \). Allospecific cytokine+ T-cell frequencies were reduced in alloanergized CD19-CAR cells to cell frequencies of 0.4% \( \pm (0.2\% \), CD4+IFN-\( \gamma \), 0.15% \( \pm (0.1\% \), CD8+IFN-\( \gamma \), 0.20% \( \pm (0.1\% \), CD4+TNF-\( \alpha \), and 0.17% \( \pm (0.02\% \), CD8+TNF-\( \alpha \). This represented median fold reductions of 11 (range, 3–17; CD4+IFN-\( \gamma \), 15 (range, 5–25; CD4+TNF-\( \alpha \), 23 (range, 1–7–19; CD8+IFN-\( \gamma \), and 26 (range, 1–8–10; CD8+TNF-\( \alpha \). In contrast, CD19-specific cytokine responses after stimulation with CD19+ Daudi and U251T targets were only modestly reduced after alloanergization. CD19-specific IFN-\( \gamma \) cell frequencies were 49% (nonanergized) and 26% (anergized; Daudi) and 50% (nonanergized) and 38% (alloanergized; CD19+ U251T), representing 1.9- and 1.3-fold reductions, respectively (Fig. 3). This showed that alloanergization preferentially reduced allospecific cytokine responses within CD19-CAR cells while maintaining the majority (but not all) of CD19-specific responses.

**Phenotypic characteristics of CD19-CAR cells after alloanergization.** Although the proportion of CD8+ CD19-CAR cells expressing surface CAR was not affected by alloanergization, the proportion of CD4+ CD19-CAR cells expressing surface CAR was temporarily reduced by up to 50%. The majority of CD19-CAR cells were CD45RO+CD27- with cell frequencies of 3.8% contained both CD4+ and CD8+ T cells, when extended determined. Majority of CD19-CAR cells were CD45RO+CD27- memory cells while maintaining the majority (but not all) of CD19-specific responses.

<table>
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<tr>
<th>CD4+</th>
<th>CD8+</th>
<th>Mitogen restimulation</th>
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<tbody>
<tr>
<td>Nonalloanergized</td>
<td>21 ( \pm (6.2% )</td>
<td>15 ( \pm (6.1% )</td>
</tr>
<tr>
<td>Alloanergized</td>
<td>6.9 ( \pm (4.7% )</td>
<td>4.1 ( \pm (3.6% )</td>
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<td>( P^* )</td>
<td>0.01</td>
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**Table 1. Percent of CD4+ and CD8+ subsets of nonalloanergized and alloanergized CD19-specific CAR+ T cells proliferating after restimulation with alloanergents or mitogenic CD3 and CD28 antibodies**

NOTE: Numbers in parentheses indicate SD.

*Two-tailed paired \( t \) test comparing values for nonalloanergized and alloanergized cells.

patterns of CD28 and Fas (CD95) to distinguish CD28+CD95+ effector memory T cells (TEM) from CD28−CD95+ central memory (TCM) cells (36), we were able to identify similar proportions of TCM (24.2% and 37.2%) and TEM (75.8% and 62.8%) cells before and after alloanergization of CD19-CAR cells. Using coexpression patterns of CD45RO and CD62L (37), CD19-CAR cells also contained similar proportions of TCM cells (31.0% and 45.7%) and TEM cells (69.0% and 53.8%) before and after alloanergization (Fig. 4A). This is consistent with CAR transgene expression in TCM cells and preservation of these cells after alloanergization.

**CD19-specific cytolytic function of CD19-CAR cells was preserved after alloanergization.** CD19-CAR cells were evaluated for redirected killing before and after alloanergization in a 4-hour \( ^{31} \text{Cr} \) release assay (Fig. 4B). CD19-CAR cells effectively lysed CD19+ B-cell lines before and after alloanergization (Daudi: before, 59.4%; after, 56.7%; NALM-6: before, 29%; after, 26.7% at an effector/target ratio of 20:1). Retention of CD19 specificity was shown by the 1.8-fold (before alloanergization) and 2.2-fold (after alloanergization) increased killing of CD19+ targets when compared with CD19+ K562 targets at an effector/target ratio of 20:1. In comparison, alloanergization resulted in a 5-fold reduction in lysis of cultured first-party allogeneic target cells at an effector/target ratio of 20:1 (Fig. 4C).

**CD19-specific proliferation of CD19-CAR cells after alloanergization.** We next compared the capacity for CAR-dependent proliferation of nonanergized and alloanergized CD19-CAR cells using CD19+ aAPC without or with CD64 (FcγRII)-loaded OKT3 (to provide an additional CD3-dependent antigen-independent proliferative signal). The numbers of nonanergized CD19-CAR cells were expanded by 3 to 4 log over 21 days on both CD19+ aAPC and OKT3-loaded CD19+ aAPC. Alloanergized CD19-CAR cells retained the capacity to expand on both CD19+ aAPC and OKT3-loaded CD19+ aAPC, with a 2- to 2.5-log expansion over 21

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days. Although CD19-dependent expansion was 1 to 2 log less than that seen in nonanergized CD19-CAR cells, the retention of capacity to proliferate in a CAR-dependent manner implies that alloanergization did not substantially interfere with the ability of the CAR to provide and sustain a proliferative signal. In contrast, alloanergized CD19-CAR cells could not be expanded by repeat stimulation with first-party allostimulators (Fig. 5A). Furthermore, expansion of alloanergized CD19-CAR cells on OKT3-loaded CD19+ aAPC restored the proportion of CD4+ CD19-CAR cells expressing surface CAR to similar levels seen in nonalloanergized cells (Fig. 5B).

Importantly, alloanergized CD19-CAR cells remained hyporesponsive to allostimulation after in vitro expansion on CD19+ aAPC (Fig. 5C). Finally, to confirm that CD19-expanded alloanergized CAR+ cells retained effector function, we again examined CD19-specific IFN-γ production. In vitro expanded alloanergized CD19-CAR cells retained up to 70% of their capacity to produce IFN-γ after contact with cell-surface CD19 when compared with expanded nonanergized CD19-CAR cells. Intracellular cytokine staining showed a 3-fold increase in IFN-γ production when alloanergized CD19-CAR cells were stimulated with a CD19+ B-cell line (Daudi). IFN-γ production

Figure 3. Alloanergization of CD19-CAR cells results in reduced allospecific cytokine production. A, cytokine secretion by nonanergized and alloanergized CD19-CAR cells after restimulation with allostimulators or SEB. Flow cytometer dot plots are shown depicting intracellular cytokine production in CD4+ and CD8+ cells, gated on CD3+ events excluding irradiated stimulator cells. Boxed regions represent cytokine+ events and numbers represent frequency of cytokine+ events expressed as a percentage of CD4+ or CD8+ cells. Results are shown for a representative experiment of three. B, frequencies of cytokine+ cells in nonanergized and alloanergized CD19-CAR cells after restimulation with allostimulators, SEB, or CD19+ target cells.

Davies et al. Cancer Res; 70(10) May 15, 2010 Cancer Research

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was 1.6-fold greater when alloanergized cells were stimulated with CD19\(^+\) transfected U251T glioma cells in comparison with CD19\(^-\) U251T cells, showing the CD19 specificity of IFN-\(\gamma\) production (Fig. 5D). These data are consistent with retention of capacity of expanded alloanergized CD19-CAR cells to be activated via the introduced CD19-CAR.

**Discussion**

Disease relapse remains a major cause of treatment failure after allogeneic HSCT, especially in patients with advanced B-cell malignancies. A significant unmet need, therefore, is a clinically applicable strategy to enhance the antitumor effect of allogeneic donor T cells. In the present study, we have developed such a strategy by combining the approaches of redirection of human donor T cells to the B-cell antigen CD19 and alloanergization to reduce the potential of such cells to mediate GvHD. We show that the strategy of alloanergization effectively reduces alloresponses without adversely affecting the CD19-specific effector functions of CD19-CAR cells. This combined approach could be used to reduce relapse without increasing toxicity after allogeneic HSCT for patients with B-lineage leukemias and lymphomas.

A major concern with the infusion of allogeneic CAR\(^+\) T cells is their potential to mediate toxicity in the form of GvHD. CAR\(^+\) T cells can be activated by pathogen-specific antigens via their endogenous \(\alpha\beta\) TCR, indicating that these receptors and their associated intracellular pathways remain functionally intact (7, 30, 38). We have previously shown that CD19-CAR cells generated using the \(S\beta\) technology retain broad endogenous \(\alpha\beta\) TCR \(V\beta\) distribution (18), in apparent contrast to some other strategies used to enrich antigen-specific T cells using repetitive antigenic stimulation (39). It has also been shown that murine and human folate-binding protein–specific CAR\(^+\) T cells can be activated via endogenous \(\alpha\beta\) TCR by stimulation with alloantigens, supporting the potential of such cells to mediate alloresponses (40). In our current study, we detected CD4\(^+\) alloprecursor frequencies within human CD19-CAR cells at comparable levels to those detected by CFSE dye dilution in unmanipulated human CD4\(^+\) T cells by Martins and colleagues (1.1%), who, in common with our strategy, used single-donor allostimulator PBMC (34). This suggests that allopregenerative CD4\(^+\) T cells occur at a similar frequency within human CD19-CAR cells and unmanipulated T cells.

Alloanergization of CD19-CAR cells effectively and specifically reduced proliferative and cytokine alloresponses in these cells. When the confounding effect of CD19-driven proliferation was removed (by using CD19-depleted allostimulators in both the alloanergization step and in assays to detect...
residual alloreactivity), the efficiency of alloanergization of CD19-CAR cells was >90%. This was consistent with both our previous published data and other effective strategies used to reduce alloreactivity of HLA-mismatched PBMC (23, 26). Because our strategy of alloanergization only directly affects CD28+ T cells, the strategy could theoretically be less effective at reducing alloresponses in CD8+ CD19-CAR cells than in CD4+ CD19-CAR cells, as human CD8+ T cells typically contain a lower proportion of CD28+ cells compared with human CD4+ T cells. However, alloanergization reduced alloproliferative responses effectively in both CD4+ and CD8+ CD19-CAR cells. This may reflect an indirect effect on CD8+ T cells consequent to alloanergization of CD4+ T cells, consistent with reports that alloproliferative CD4+ T cells are required for alloreactive CD8+ T cells to proliferate (34, 41). The concern remains that CD4+CD28neg TEM-mediated alloresponses may persist after alloanergization. However, clinically significant GvHD mediated by such cells is likely to be limited. TEm cells are less potent mediators of proliferative alloresponses in vitro than TCM or naïve human T cells (42), and human CD28neg T cells typically have shortened telomeres compared with CD28+ T cells (43), predicting a restricted life span in vivo after infusion (44).

Alloanergization did not reduce the proportion of CD8+ CD19-CAR cells expressing surface CAR, and CD19-specific redirected target cell cytolysis was preserved, demonstrating retained functionality and specificity. However, alloanergization resulted in modest reductions in the proportion of CD4+ CD19-CAR cells expressing surface CAR, frequencies of CD19-specific IFN-γ+ cells, and capacity for CD19-CAR cells to expand in vitro after stimulation on CD19+ aAPC. These findings suggest that the in vivo efficacy of alloanergized
CD19-CAR cells could be reduced in comparison with nonanergized cells. However, more than half of CD4+ CD19-CAR cells expressing cell-surface CAR and 50% to 70% of the proportion of CD19-CAR cells secreting IFN-γ after CD19 stimulation were retained after alloanergization, and CD19-specific proliferation was still demonstrable, consistent with retention of significant capacity for CD19-specific expansion and target cell lysis. Furthermore, it is likely that homeostatic expansion resulting from the lymphopenic environment created after allogeneic HSCT would augment expansion and persistence of infused alloanergized donor CD19-CAR cells. This is supported by the data from in vitro expansion of alloanergized CD19-CAR cells on CD19-aAPC loaded with OKT3, which restored the proportion of CD4+ CD19-CAR cells expressing surface CAR to levels seen in nonalloanergized cells without loss of allospecific hyporesponsiveness, suggesting that CD19-specific function and alloenery might be maintained after expansion. Further studies using an immunodeficient tumor-bearing mouse model could be used to compare the in vitro persistence and antitumor efficacy of nonanergized and alloanergized human CD19-CAR cells.

Allogeneic CD19-CAR cells could be used to augment antitumor effects in a variety of allogeneic HSCT settings. These data support the application of a clinical strategy in which CD19-CAR cells are generated from allogeneic donors and subsequently anergized to recipient alloantigens before infusion after allogeneic HSCT. The broad endogenous TCR Vβ subfamily distribution retained by CD19-CAR cells generated using the SB system (18) suggests that these cells could also contribute to pathogen-specific immunity after allogeneic HSCT via their endogenous αβ TCR. Therefore, allogeneic HSCT approaches using T-cell–depleted hematopoietic stem cell sources or umbilical cord blood cells, both of which are associated with delayed immune reconstitution and increased infectious complications (45–47), would be particularly suitable platforms for the use of allogeneic donor CD19-CAR cells. Although we are developing approaches to limit off-target effects, one consequence of allogeneic CD19-CAR T-cell therapy might be destruction of healthy donor–derived CD19+ B cells. Intravenous immunoglobulin could be used to correct clinically significant hypogammaglobulinemia in such an event. Another potential limitation to our approach is that repeated in vivo stimulation may contribute to replicative senescence of CD19-CAR cells via telomere erosion (48) and preclude their long-term persistence. In this case, repeat infusions of alloanergized CD19-CAR cells could provide a persistent antitumor effect.

In summary, we describe the successful application of alloanergization to selectively reduce alloreactivity in human CD19-specific T cells without significant impairment of CAR-dependent effector functions. Nonviral gene transfer of CAR, propagation on aAPC, and induction of alloenery all use methods currently individually in use in phase 1/2 clinical trials. These approaches could therefore be readily applied in combination at a clinical scale to generate donor-derived T cells engineered to contain enhanced antitumor activity, but reduced alloreactivity, suitable for use after allogeneic HSCT to reduce disease relapse while limiting toxicity from GvHD.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Davies et al.


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