T Cells Expressing CD19/CD20 Bispecific Chimeric Antigen Receptors Prevent Antigen Escape by Malignant B Cells

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Abstract

The adoptive transfer of T cells expressing anti-CD19 chimeric antigen receptors (CARs) has shown remarkable curative potential against advanced B-cell malignancies, but multiple trials have also reported patient relapses due to the emergence of CD19-negative leukemic cells. Here, we report the design and optimization of single-chain, bispecific CARs that trigger robust cytotoxicity against target cells expressing either CD19 or CD20, two clinically validated targets for B-cell malignancies. We determined the structural parameters required for efficient dual-antigen recognition, and we demonstrate that optimized bispecific CARs can control both wild-type B-cell lymphoma and CD19- mutants with equal efficiency in vivo. To our knowledge, this is the first bispecific CAR capable of preventing antigen escape by performing true OR-gate signal computation on a clinically relevant pair of tumor-associated antigens. The CD19-OR-CD20 CAR is fully compatible with existing T-cell manufacturing procedures and implementable by current clinical protocols. These results present an effective solution to the challenge of antigen escape in CD19 CAR T-cell therapy, and they highlight the utility of structure-based rational design in the development of receptors with higher-level complexity.

Cancer Immunol Res; 4(6); 498–508. ©2016 AACR.

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Introduction

Adoptive T-cell therapy has demonstrated clinical efficacy against advanced cancers (1, 2). In particular, multiple clinical trials have shown that T cells programmed to express anti-CD19 chimeric antigen receptors (CARs) have curative potential against relapsed B-cell malignancies (3–9). However, these trials have also revealed critical vulnerabilities in current CAR technology, including susceptibility to antigen escape by tumor cells (7, 10). For example, a recent trial of CD19 CAR T-cell therapy revealed that 90% of patients achieved a complete response, but 11% of those patients eventually relapsed with CD19-negative tumors (10). Antigen escape has also been observed in the adoptive transfer of T cells expressing NY-ESO1-specific T-cell receptors (11) and in cancer vaccine therapy for melanoma (12, 13).

The probability of antigen escape by spontaneous mutation and selective expansion of antigen-negative tumor cells decreases with each additional antigen that can be recognized by the CAR T cells. Therefore, a potential prophylaxis against antigen escape is to generate T cells capable of recognizing multiple antigens. Here, we present the rational design and systematic optimization of single-chain, bispecific CARs that efficiently trigger T-cell activation when either of two pan-B-cell markers, CD19 or CD20, is present on the target cell. These CARs can be efficiently integrated into primary human T cells and administered in the same manner as CAR T cells currently under evaluation in the clinic.

A single-chain, bispecific CAR targeting CD19 and the human epidermal growth factor receptor 2 (HER2/neu) was previously reported by Grada and colleagues (14). This bispecific receptor, termed TanCAR, efficiently triggered T-cell activation in response to either CD19 or HER2. However, because CD19 and HER2 are not typically expressed on the same cell, the TanCAR remains a proof-of-concept design. Importantly, Grada and colleagues highlighted that the TanCAR is less strongly activated both in vitro and in vivo when challenged with target cells that express HER2 alone, as compared with HER2/CD19 double-positive targets (14). This observation suggests that tumor cells can still escape TanCAR detection by eliminating CD19 expression.

To effectively prevent antigen escape, the bispecific CAR must not only recognize two antigens, but also process both signals in a true Boolean OR-gate fashion—i.e., either antigen input should be sufficient to trigger robust T-cell output. We thus refer to this particular type of bispecific receptors as “OR-gate CARs.” Here, we report on the development of CD19-OR-CD20 CARs, which trigger robust T-cell-mediated cytokine production and cytotoxicity when either CD19 or CD20 is present on the target cell. We demonstrate that the size and rigidity of CAR molecules can be
calibrated to match the specific antigens targeted, and the optimal OR-gate CAR structure can be deduced from known structural requirements for single-input CARs. Finally, we show that the CD19-OR-CD20 CARs can control both wild-type and CD19\* mutant B-cell lymphomas with equal efficiency in vivo, thus providing an effective safeguard against antigen escape in adoptive T-cell therapy for B-cell malignancies.

**Materials and Methods**

**Plasmid construction**

Bispecific CD19-CD20 CARs were constructed by isothermal assembly (15) of DNA fragments encoding the CD19 single-chain variable fragment (scFv) derived from the FMC63 mAb (16), the CD20 scFv derived from the Leu-16 mAb (17), an IgG4-based extracellular spacer, the CD28 transmembrane domain, and the cytoplasmic domains of 4-1BB and CD3ζ. Sequences of extracellular spacers and linkers connecting scFv domains are listed in Supplementary Table S1, and sequences of all OR-gate CARs are available in GenBank (accession numbers KX000905 to KX000911). All CARs were fused to a truncated epidermal growth factor receptor (EGFRt) via a T2A peptide to facilitate antibody staining and sorting of CAR-expressing cells (18).

**Cell line generation and maintenance**

Parental Raji cells were obtained from the ATCC in 2003, and parental K562 cells were a gift from Dr. Laurence Cooper in 2001. Both cell lines were authenticated by short tandem repeat profiling at the University of Arizona Genetics Core in 2015. The Epstein–Barr virus-transformed lymphoblastoid cell line (TM-LCL) was made from peripheral blood mononuclear cells as previously described (19). CD19\*, CD20\*, and CD19\*/CD20\* K562 cells were generated by lentivirally transducing parental K562 cells with CD19 and/or CD20 constructs. CD19\* Raji cells were generated by CRISPR/Cas9-mediated gene editing. Raji cells (2 × 10⁶) were transiently transduced with CD19-CRISPR-T2A-NM plasmid (2 μg) using the Amazuka Nucleofection Kit V (Lonza) and the Amazuka Nucleofector 2b Device (Lonza). After 24 hours, cells were coincubated with G418 sulfate (1.5 mg/mL; Enzo Life Sciences) for 11 days and further expanded in the absence of antibiotics for 11 days. CD19\* cells were enriched by magnetic bead–based cell sorting. A pure CD19\* population was subsequently obtained by fluorescence-activated cell sorting using the FACSARia (II) at the UCLA Flow Cytometry Core Facility. All TM-LCL, Raji, and K562 cell lines were maintained in complete T-cell medium [RPMI-1640 (Lonza) with 10% heat-inactivated FBS (HI-FBS; Life Technologies)] and human embryonic kidney 293T cells (ATCC) were cultured in DMEM (HyClone) supplemented with 10% HI-FBS.

**Generation of CAR-expressing primary human T cells**

CD8\+/CD4\+/CD62L\* T cells were isolated from healthy donor whole blood obtained from the UCLA Blood and Platelet Center, stimulated with CD3/CD28 T-cell activation Dynabeads (Life Technologies) at a 1:1 bead:cell ratio, and lentivirally transduced 72 hours later at a multiplicity of infection of 1.5. All T cells were expanded in complete T-cell medium supplemented with penicillin–streptomycin (100 U/mL; Life Technologies) and fed IL2 (50 U/mL; Life Technologies) and IL15 (1 ng/mL; Miltenyi) every 48 hours. Dynabeads were removed 9 or 10 days after isolation. CAR\+ cells were enriched by magnetic bead–based sorting (Miltenyi) and expanded by stimulation with irradiated TM-LCLs at a T-cell:TM-LCL ratio of 1:7. Mock-transduced T cells were stimulated using the rapid expansion protocol as previously described (20). Each in vitro experiment was repeated with T cells from different donors (T cells were never pooled). See Supplementary Materials and Methods for additional details.

**Cytotoxicity assay**

Target cells (K562 cells) seeded at 1 × 10⁴ cells/well in a 96-well plate were coincubated with effector cells at varying effector-to-target (E:T) ratios in complete media without phenol red and with 5% HI-FBS for 4 hours. Supernatants were harvested and analyzed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega).

**Cytokine production quantification**

Target cells were seeded at 5 × 10⁴ cells/well in a 96-well plate and coincubated with effector cells at an E:T ratio of 2:1 for 24 hours. Cytokine concentrations in the culture supernatant were measured with the BD Cytometric Bead Array Human Th1/Th2 Cytokine Kit II (BD Biosciences).

**In vivo xenograft studies in NOD/SCID/γc\−/− (NSG) mice**

All in vivo experiments were approved by the UCLA Institutional Animal Care and Use Committee. Six- to eight-week-old female NSG mice were bred in house by the UCLA Department of Radiation and Oncology. EGFP\+, firefly luciferase (fLuc)–expressing Raji cells (5 × 10⁴) were administered to NSG mice via tail-vein injection. Seven days later, mice bearing engrafted tumors were treated with 10 × 10⁶ mock-transduced or CAR\+/EGFRt cells via tail-vein injection. Tumor progression was monitored by bioluminescence imaging using an IVIS Lumina III LT Imaging System (PerkinElmer). Peripheral blood was obtained by retro-orbital bleeding 10 days and 20 days after tumor-cell injection, and samples were analyzed by flow cytometry.

**Statistical analysis**

Statistical significance of in vitro results was analyzed using two-tailed, unpaired, homoscedastic Student t test. Survival curves were evaluated by the log-rank test, and statistical significance was determined by comparing log-rank test statistics against a χ² table with the degree of freedom equal to one.

**Results**

**CD19 and CD20 CARs require distinct extracellular spacer lengths**

Conventional CAR molecules comprise four main domains: an scFv, an extracellular spacer, a transmembrane domain, and a cytoplasmic tail including costimulatory signals and the CD3ζ chain (Fig. 1A). The CD19 CAR has superior activity when constructed with a short extracellular spacer (21), but no systematic study has been reported for CD20 CARs. Therefore, we constructed and characterized a series of CD20 CARs incorporating long (IgG4 hinge-CH2-CH3; 229 aa), medium (IgG4 hinge-CH3; 119 aa), or short (IgG4 hinge; 12 aa) extracellular spacers connecting the Leu-16 scFv to the CD28 transmembrane domain (Supplementary Fig. S1A). All three CARs were efficiently expressed in primary CD8\+ human T cells [Supplementary...
When challenged with CD20⁺ target cells, T cells expressing the long-spacer CAR consistently showed greater target-cell lysis, cytokine production, and T-cell proliferation (Supplementary Fig. S1C–S1E), indicating functional superiority of the long-spacer CAR for CD20 detection.

Bispecific and single-input CARs share antigen-specific structural requirements

Second-generation, CD19-OR-CD20 CARs were constructed by taking the standard four-domain CAR architecture and incorporating two scFvs connected in tandem via a G4S flexible linker (Fig. 1B). In light of the different preferences in extracellular spacer length for CD19 and CD20 single-input CARs, a panel of CD19-OR-CD20 CARs was constructed to systematically evaluate the effects of spacer length and the ordering of the two scFv domains (Fig. 1C). Each CAR was constructed in the scFv #1 (VL-VH) – scFv #2 (VH-VL) orientation to minimize potential mispairing of VL and VH domains between the two scFvs. All four OR-gate CARs were produced at full length and expressed on the surface of primary human CD8⁺ T cells (Supplementary Fig. S2A and S2B).

When challenged with CD19⁺ target cells, the OR-gate CARs showed superior cytokine production and target-cell lysis when a short extracellular spacer was used, regardless of scFv domain order (Fig. 2). Both long-spacer CARs failed to produce IL2 (Fig. 2A) and had minimal lysis activity against CD19⁺ targets (Fig. 2B). Thus, short-spacer CARs were more effective in targeting CD19, consistent with previous reports of single-input CD19 CARs (21).

Although the long-spacer CARs did not target CD19, they could respond to CD20 (Fig. 2A). The 20-19 short CAR, which contained a short spacer and the CD20 scFv in the membrane-distal position, also performed well in cytokine production and was the most efficient at lysing CD20⁺ targets (Fig. 2). Given the scFv orientation in this construct, the CD19 scFv and the G4S linker between the two scFv domains could serve as a proxy spacer that projects the CD20 scFv away from the T-cell membrane, to a position conducive for CD20 binding. We thus hypothesized that modifying the G4S linker while keeping the 20-19 short CAR configuration might further enhance CD20 response without compromising the receptor’s sensitivity toward CD19⁺ target cells.

Sequence modifications on scFv linkers enable effective targeting of disparate antigens

A new panel of 20-19 short CARs was constructed with various linker sequences inserted between the two scFv domains (Fig. 3A). The original, short (G4S)₁ flexible linker was compared against a long (G4S)₄ flexible linker, a short (EAAAK)₁ rigid linker, and a long (EAAAK)₃ rigid linker (22, 23). CARs with modified linker sequences were expressed at full length and localized to the cell surface (Supplementary Fig. S2C and S2D).

To evaluate the utility of OR-gate CARs in preventing antigen escape, a mutant CD19⁺ lymphoma cell line was generated by
CRISP/Cas9-mediated genome editing of Raji lymphoma cells (Supplementary Fig. S3). As expected, the single-input CD19 CAR-T cells showed no response to CD19⁺ target cells (Fig. 3B–D). In contrast, T cells expressing OR-gate CARs efficiently lysed both wild-type (WT; CD19⁺/CD20⁻) and CD19⁺ target cells (Fig. 3D).

The original OR-gate CAR with a (G4S)₁ linker had lower toxicity against mutant (CD19⁺/CD20⁻) Raji compared with WT Raji, indicating suboptimal CD20 targeting. Increasing the length and/or rigidity of the linker sequence improved the OR-gate CARs’ ability to recognize CD20, resulting in...
equally efficient elimination of both WT and CD19⁺ Raji target cells (Fig. 3D).
In addition to enhanced cytotoxicity, modified OR-gate CARs expressed more activation and degranulation markers, and they produced significantly more IFNγ, TNFα, and IL2 compared with the original CAR with a (G4S)1 linker (Fig. 3B and C). The OR-gate CAR with a (G4S)4 linker showed similar levels of effector output compared with the single-input CD20 CAR (Fig. 3B–D). Thus, linker modifications successfully compensated for impairments in CD20 targeting imposed by the short extracellular spacer, which was necessary for efficient CD19 targeting.

The increase in CD20 targeting efficiency obtained by linker modifications did not compromise CD19 targeting capability. All 20-19 short CARs, regardless of linker type, showed robust CD69, CD137, and CD107a expression when challenged with WT Raji or CD19⁺/CD20⁻ K562 target cells (Supplementary Fig. S4A). Compared with the single-input CD19 CAR, OR-gate CARs triggered comparable expression of activation and degranulation markers, as well as IFNγ production in response to CD19 stimulation (Supplementary Fig. S4A and S4B). The OR-gate CAR T cells could lyse CD19⁺ target cells as efficiently as single-input CD19 CAR T cells (Supplementary Fig. S4C). Taken together, these results demonstrate that OR-gate CARs can efficiently detect and lyse CD19⁺ escape mutants in vitro, and that rational modifications to the CAR structure successfully improved CD20 targeting while maintaining high CD19 sensitivity.

**Bi- and tri-specific CARs**

The increase in CD20 targeting efficiency obtained by linker modifications did not compromise CD19 targeting capability. All 20-19 short CARs, regardless of linker type, showed robust CD69, CD137, and CD107a expression when challenged with WT Raji or CD19⁺/CD20⁻ K562 target cells (Supplementary Fig. S4A). Compared with the single-input CD19 CAR, OR-gate CARs triggered comparable expression of activation and degranulation markers, as well as IFNγ production in response to CD19 stimulation (Supplementary Fig. S4A and S4B). The OR-gate CAR T cells could lyse CD19⁺ target cells as efficiently as single-input CD19 CAR T cells (Supplementary Fig. S4C). Taken together, these results demonstrate that OR-gate CARs can efficiently detect and lyse CD19⁺ escape mutants in vitro, and that rational modifications to the CAR structure successfully improved CD20 targeting while maintaining high CD19 sensitivity.

**Bi-specificity does not affect CAR T-cell growth, differentiation, exhaustion profile, or lytic capability in vitro**

Despite comparable activation marker expression and lytic capabilities, CD19 and OR-gate CAR T cells showed disparate IL2 production upon antigen stimulation (Supplementary Fig. S4B). To investigate potential effects of this difference in cytokine production, we examined the cell differentiation pattern and proliferation rate of the various CAR T-cell lines during extended coinoculation with Raji target cells in the absence of exogenous cytokines.

Across multiple donors, we consistently observed a slight but statistically significant elevation of central memory (Tcm), and that rational modifications to the CAR structure successfully improved CD20 targeting while maintaining high CD19 sensitivity.

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antigen-stimulated IL2 production does not have a major impact on cell-type differentiation in vitro. Furthermore, CD19 and OR-gate CAR T cells had similar proliferation rates upon antigen stimulation (Fig. 4B). Thus, the amount of IL2 produced by OR-gate CAR T cells is sufficient to support robust T-cell proliferation, an observation that was later confirmed in vivo.

Despite producing similar IL2 concentrations as OR-gate CAR T cells, CD20 CAR T cells showed significantly weaker proliferation compared with CD19 CAR T cells (Fig. 4B). When single-input CD19 and CD20 CART-cell lines were mixed and cocultured with WT Raji target cells, the CD19 CART cells proliferated significantly more than CD20 CART cells, ultimately leading to a net decrease in the CD20 CART-cell population (Fig. 4C). Thus, simultaneous administration of a mixture of two CART-cell lines can result in the selective expansion of one at the expense of the other. This behavior highlights an important benefit of using a single, bispecific CAR T-cell product that can ensure the maintenance of both CD19 and CD20 recognition capabilities while avoiding growth competition between multiple T-cell products.

Figure 5. OR-gate CAR T cells maintain robust lysis capability through repeated antigen stimulation. Mock-transduced and CAR T cells were coincubated with WT Raji targets for 6 days, and then coincubated with CD19–mutant Raji targets for another 6 days. A, survival of WT Raji cells coincubated with effector T cells. B, survival of CD19–Raji cells coincubated with T cells that were previously challenged with WT Raji cells. (Note: Mock-transduced T cells had been overwhelmed by WT Raji by Day 6 and were not rechallenged with mutant Raji.) C, exhaustion marker staining of CAR T cells before antigen stimulation, 48 hours after coincubation with WT Raji (Day 2), and 48 hours or 6 days after subsequent coincubation with CD19–Raji cells (Days 8 and 12, respectively). At each time point, cells were surface-stained for Lag-3, Tim-3, and PD-1 and then analyzed for the simultaneous expression of one, two, or three markers. Values shown are the mean of triplicates, with error bars indicating one SD. Results are representative of three independent experiments using CAR T cells derived from three different donors.
(Fig. 5A), at which point each sample was rechallenged with CD19− mutant Raji cells at a 2:1 E:T ratio. Target-cell count indicated that both OR-gate and CD20 CAR T cells efficiently eliminated CD19− targets, whereas CD19 CAR T cells failed to curb the outgrowth of escape mutants (Fig. 5B). Surface antibody staining revealed consistent upregulation of PD-1, Tim-3, and Lag-3 upon antigen stimulation across all CAR constructs (Fig. 5C; Supplementary Fig. S5). Upon subsequent challenge with CD19− target cells, exhaustion marker expression was sustained in OR-gate and CD20 CAR T cells but declined in CD19 CAR T cells (Fig. 5C; Supplementary Fig. S5). Taken together, these results indicate that OR-gate CAR T cells retain robust effector function upon repeated antigen presentation, and they exhibit equivalent lysis capabilities and exhaustion marker expression patterns as CD20 CAR T cells in response to CD19− escape mutants.

OR-gate CARs prevent tumor antigen escape in vivo

To determine the in vivo functionality of OR-gate CARs, NOD/SCID/γc−/− (NSG) mice were injected with either a pure population of WT Raji cells or a 3:1 mixture of WT and CD19− Raji cells. Mice bearing established xenografts were then treated with CD8+ CAR T cells. As expected, single-input CD19 CAR T cells significantly extended the survival of animals engrafted with WT Raji cells but failed to control the mixed Raji population (Fig. 6; Supplementary Fig. S6A). Postmortem analysis revealed the outgrowth of CD19− mutants in the mixed Raji xenograft, confirming antigen escape from single-input CAR T-cell therapy (Supplementary Fig. S6B).

In contrast with the single-input CD19 CAR, OR-gate CARs could target WT and mixed Raji tumors with equal efficiency (Fig. 6; Supplementary Fig. S6C), demonstrating that OR-gate CAR T cells were unaffected by the loss of CD19 expression on tumor cells and only require a single antigen to trigger robust antitumor functions. The OR-gate CARs were as effective as the single-input CD19 CAR in controlling the growth of WT Raji cells (Supplementary Fig. S6D), confirming that the broadening of antigen specificity did not compromise in vivo antitumor efficacy against CD19− targets. Taken together, these results demonstrate that OR-gate CARs can efficiently target malignant B cells and abrogate the effects of tumor antigen loss in vivo.

Although OR-gate CARs were unaffected by the loss of CD19 antigen, none of the CAR T-cell lines—including second-generation CD19 CAR T cells challenged with WT Raji cells—could completely eradicate engrafted tumors under the conditions tested. Analysis of retro-orbital blood samples obtained 3 days after T-cell injection confirmed the presence of CAR T cells.

**Figure 6.** OR-gate CARs abrogate the effects of antigen escape in vivo. A, tumor progression in NSG mice bearing WT or mixed (75% WT, 25% CD19−) Raji xenografts. Bioluminescence imaging was performed on days 6, 18, and 21 after tumor injection (T cells were injected on day 7). B, survival of mice bearing mixed Raji tumor xenografts and treated with T cells expressing no CAR, the single-input CD19 CAR, or OR-gate CARs. n = 5 in all test groups. P values were calculated by log-rank test analysis; n.s., not statistically significant (P > 0.1); *, P < 0.1; **, P < 0.05. Results represent one independent trial.
Discussion

CD19 CAR T-cell therapy has yielded remarkable clinical outcomes in the treatment of acute and chronic B-cell malignancies (3–9). However, this treatment strategy’s vulnerability to antigen escape has been highlighted by multiple cases of relapse resulting from the emergence of CD19− tumor cells (7, 10). To arm CD19 CAR T cells against antigen escape, we constructed novel CARs capable of OR-gate signal processing—i.e., receptors that can trigger robust T-cell responses as long as the target cells express either CD19 or CD20. Results of our in vitro and in vivo characterization experiments indicate that the CD19-OR-CD20 CARs can safeguard against the effects of antigen escape by targeting malignant B cells through CD20 when CD19 expression has been lost.

The choice of CD19 and CD20 as the target-antigen pair provides several important advantages. First, CD19 and CD20 are both clinically validated B-cell antigens expressed on the vast majority of malignant B cells (10, 25, 26), making the CD19-OR-CD20 CAR a clinically applicable construct that addresses a real challenge facing adoptive T-cell therapy. Second, efforts to broaden the recognition capability of CAR T cells are often met with the undesirable side effect of increased on-target, off-tumor toxicity. However, this trade-off does not apply to the case of CD19 and CD20, because both are exclusively expressed on B cells and have the same off-tumor toxicity profile. Finally, the ubiquitous expression of CD19 and CD20 on B cells and their known and predicted roles in promoting B-cell survival (27, 28) suggest that simultaneous loss of both antigens would be a very low-probability event. Therefore, targeting CD19 and CD20 is expected to provide an effective safeguard against antigen escape by malignant B cells.
In principle, multiple receptor configurations can be adopted to achieve bispecific signal computation, such as coexpressing two different CARs in one T cell (29) or mixing two CAR T-cell lines, each targeting a different antigen (30). Instead, we chose to engineer dual-antigen recognition capability into a single CAR molecule due to a number of advantages. First, compared with expressing two separate single-input CARs in one T cell, the bispecific CAR has a significantly smaller DNA footprint (reduced by ~40% in DNA length), and previous studies have shown that construct size significantly affects viral vector packaging and transduction efficiency (31, 32). Most clinical T-cell products are administered as a polyclonal population without prior sorting for CAR+ cells (3, 5, 6), thus the ability to achieve high transduction efficiency has a direct impact on clinical efficacy. Genetic compactness becomes particularly critical when features such as suicide genes (33) and additional signal-processing units (e.g., inhibitory receptors; ref. 34) also need to be integrated into the CAR T cells to ensure safety or enhance efficacy. Second, compared with mixing two different single-input CAR T-cell lines, the ability to produce and administer a single, bispecific CAR T-cell product significantly reduces treatment costs and increases the probability of successful T-cell production within a short clinical timeframe. Third, the CD19 CAR has outperformed all other CARs evaluated in the clinic to date, including the CD20 CAR (35, 36). Our data demonstrate that in a coculture, CD19 CAR T cells have a significant growth advantage over CD20 CAR T cells, resulting in a net decline in CD20 CAR T-cell count despite the presence of CD20 antigen (Fig. 4C). Therefore, it is probable that coadministering two single-input CAR T-cell populations would result in the disproportionate expansion of CD19 CAR T cells at the expense of CD20 CAR T cells, thereby compromising this strategy’s ability to safeguard against CD19 mutants when they emerge later in the treatment period. By making each T cell capable of bispecific antigen recognition, the OR-gate CAR design maximizes the number of T cells that can recognize an escape mutant when it appears. For these reasons, we chose to engineer a single CAR molecule capable of dual-antigen recognition by attaching two tandem scFv domains to the standard CAR chassis. Our data indicate that T cells expressing OR-gate CARs are indeed insensitive to the loss of CD19 on target cells, proliferate robustly in response to either CD19 or CD20 stimulation, do not exhibit altered cell-type differentiation patterns compared with single-input CAR T cells, and retain robust target-cell lysis capability upon repeated antigen stimulation.

CARs are frequently thought of as modular proteins with sensor domains (i.e., the scFv) that can be easily changed to alter receptor specificity. However, results from a previous report (21) and our own investigation (Supplementary Fig. S1) revealed that efficient CD19 and CD20 targeting by single-input CARs requires distinct receptor structures. These divergent preferences may be a consequence of structural differences between the two antigens: CD19 is an immunoglobulin-like molecule that belongs to a family of single-pass transmembrane proteins that project outward from the cell membrane (37); CD20 is a multi-pass transmembrane protein that lies close to the cell surface (38). To achieve the optimal conjugation distance between a T cell and its target, the receptor needs to be adjusted to match the size of the target antigen, resulting in the need for a shorter CAR when targeting antigens with extensive extracellular domains and vice versa. Although this hypothesis is consistent with our observations, quantitative imaging studies would be required to confirm its accuracy.

Based on our understanding of the structural requirements for productive CAR/antigen interactions, we arrived at the optimal structure for a bispecific CAR through systematic, rational design. By adjusting the extracellular spacer length and linker sequence between the two scFv domains, we were able to independently optimize CD19 and CD20 targeting without compromising the CAR’s ability to recognize either antigen. In vitro and in vivo results demonstrate that the OR-gate CARs are as efficient in CD19 targeting as the clinically successful single-input CD19 CAR, and the OR-gate CARs succeed in controlling CD19+ mutant lymphoma cells where the single-input CAR fails.

A potential concern with the design of bispecific CARs is the increased number of peptide fusion points in the receptor protein. However, almost all of the components used in the OR-gate CARs presented in this study have been tested in the clinic, including the two scFv domains, the long and short extracellular spacers, as well as the transmembrane and cytoplasmic signaling domains (3–5, 7, 8, 39). Long, flexible linker peptides consisting of glycine and serine residues have been used in fusion peptides such as rVIIa-FP, which is now being evaluated in a clinical trial (40, 41). It remains possible that rigid peptide linkers or new combinations of previously tested peptide components could result in immunogenicity. However, available evidence suggests that the probability is low and will have to be verified through extensive testing in preclinical and clinical studies.

Several studies have explored the contribution of individual CAR components to T-cell functionality, with results suggesting that the scFv framework regions, extracellular spacer length, and costimulatory signals all play important and nonobvious roles in enabling robust T-cell-mediated response to tumor antigens (21, 36, 42–45). It remains possible that additional modifications to each of these domains could further increase the OR-gate CAR’s functionality. Increasingly detailed mechanistic understanding of CAR signaling and the systematic incorporation of such knowledge into the rational design of next-generation CAR molecules will continue to facilitate the development of more effective and predictable cell-based therapeutics.

Adaptive T-cell therapy has been hailed as one of the most promising advancements in cancer therapy in recent years, and CD19 CAR T-cell therapy holds the spotlight as the most successful adoptive T-cell therapy to date. The work reported here presents an effective and clinically applicable solution to the challenge of antigen escape, which has been observed in multiple clinical trials of CD19 CAR T-cell. The CD19-OR-CD20 CAR is fully compatible with current T-cell manufacturing processes, does not impose extra burden in the form of large viral packaging and transduction payloads, and enables a single T-cell product to target two clinically validated antigens associated with B-cell leukemia and lymphoma. Finally, the design principles highlighted in this study can be further utilized to construct novel CARs for additional antigen pairs to broaden the applicability and increase the efficacy of T-cell therapy for cancer.

Disclosure of Potential Conflicts of Interest

M.C. Jensen reports serving as a scientific advisory board member and as a consultant for Juno Therapeutics, Inc., of which he is a scientific co-founder with
ownership interest (including patents). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: M.C. Jensen, Y.Y. Chen

Development of methodology: E. Zah, A. Silva-Benedict, M.C. Jensen, Y.Y. Chen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Zah, M.V. Lin, Y.Y. Chen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Zah, M.C. Jensen, Y.Y. Chen

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Acknowledgments

The authors thank Lisa Rolcynski for her technical assistance.

Grant Support

This research was funded by the NIH (1DP5OD012133, grant to Y.Y. Chen; 2R01CA136551-06A1, grant to M.C. Jensen). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 10, 2015; revised February 19, 2016; accepted March 6, 2016; published OnlineFirst April 8, 2016.

Bispeciﬁc T Cells Prevent B-cell Antigen Escape
ADDENDUM: T Cells Expressing CD19/CD20 Bispecific Chimeric Antigen Receptors Prevent Antigen Escape by Malignant B Cells
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In this article (Cancer Immunol Res 2016;4:498–508), which appeared in the June 2016 issue of Cancer Immunology Research, we reported the design and optimization of bispecific, OR-gate chimeric antigen receptors (CARs) that can trigger robust T-cell activation in response to target cells that present either CD19 or CD20 (1), thus preventing malignant B cells from escaping T-cell therapy through loss of CD19 expression. In our original study, although OR-gate CAR T cells could significantly delay tumor expansion and prolonged survival, tumors were not eradicated. Tumor persistence was not due to the loss of both CD19 and CD20 antigens, nor due to the inability of the adoptively transferred T cells to survive or expand in vivo (1). We concluded that the failure to eradicate tumors was due to suboptimal tumor or T-cell dosing and/or the aggressiveness of the Raji tumor model.

As the Raji tumor model had been used in other studies at dosages comparable with those employed in our own experiments (2), complete tumor clearance should be possible in this xenograft model using adoptively transferred CAR T cells. Here, we report follow-up studies showing that the viability levels of CAR T cells, both immediately after thawing and after 1–2 days of in vitro culture, are an important indicator of the CAR T cells’ antitumor capability in vivo. Healthy T cells with OR-gate CARs could efficiently eradicate established lymphoma xenografts even if the lymphoma cells had spontaneously lost CD19 expression, whereas single-input CD19 CAR T cells succumbed to the selective expansion of CD19 mutants.

Stability of CAR T-cell viability varies across donors
The in vivo protocol employed in our study called for the tail-vein injection of luciferase-expressing Raji tumor cells, followed by the tail-vein injection of CAR T cells upon confirmation of tumor establishment, which was defined as clear tumor signal observed on two consecutive days via bioluminescence imaging (BLI). The CAR T cells used in these studies were previously frozen and thawed on the day of injection, and cells with >70% viability were considered suitable for adoptive transfer. To better characterize the CAR T cells (from donor A) used in our initial animal study reported in (1), we thawed additional stocks of the same donor’s cells (donor A) and compared them with CAR T cells derived from other healthy donor blood samples (donors B and C) for cell viability, T-cell subtype distribution, and exhaustion marker expression. T-cell subtype distribution, exhaustion marker expression, and viability at the time of thawing were similar for all donors (Fig. 1). However, cell viability of CAR T cells from donors A and B drastically declined after 24 hours in culture, whereas donor C cells remained >70% viable, indicating that donor A cells may not have been of optimal quality (Fig. 1C). Although we had confirmed that donor A CAR T cells could survive and expand in vivo (1), they may not have proliferated well enough to completely eliminate the tumor xenograft.

CAR T cells with high post-thaw viability efficiently eradicate established tumor xenografts
To investigate this hypothesis, we repeated the in vivo study using donor C cells, which showed superior viability in culture after thawing (Fig. 1C). As in the previous study, 5 × 10⁶ Raji cells were delivered by tail-vein injection and allowed to engraft prior to treatment with 10 × 10⁶ CAR T cells from donor C. Animals were injected with either purely wild-type (WT; CD19⁺/CD20⁺) Raji cells or a mixture of 75% WT and 25% CD19⁻/CD20⁻ mutant Raji tumors. In contrast to the results obtained with donor A cells, we observed complete clearance of WT tumors treated with both single-input and OR-gate CAR T cells (Fig. 2A and B). Only OR-gate CAR T cells, however, could eradicate mixed (75% WT, 25% CD19⁻) Raji tumors (Fig. 2A and B). Animals engrafted with mixed Raji cells and treated with single-input CD19 CAR T cells eventually succumbed to tumor growths that consisted almost exclusively of the CD19⁻/CD20⁻ phenotype (Fig. 2C), confirming the selective expansion of antigen-negative clones when treated with single-input CAR T cells. Thus, unlike single-input CD19 CAR T cells, OR-gate CAR T cells limited the escape of CD19⁻/CD20⁻ mutant tumors.

OR-gate CARs prevent the spontaneous emergence of CD19⁻ mutant tumors
Having confirmed the ability to eradicate both WT and CD19-knockout tumors using high-quality CAR T cells, we next investigated whether OR-gate CAR T cells would have superior resistance compared with single-input CD19 CAR T cells against the spontaneous loss of CD19 expression by tumor cells. Specifically, we investigated whether antigen loss is dependent on the timing of CAR T-cell treatment relative to the size of tumor burden, and whether the OR-gate CAR T cells could safeguard against the spontaneous emergence of CD19⁻ tumors.

Mice were injected with 5 × 10⁵ WT Raji cells, which were allowed to grow either until first confirmation of engraftment (i.e., yielding clear tumor signal observed on 2 consecutive days via BLI, which occurred on day 6 in this set of studies), or until the tumor signal reached a 6-fold increase in radiance intensity compared with the first group (which occurred on day 9). Animals were then treated with 10 × 10⁶ donor C T cells expressing either the single-input CD19 CAR or the OR-gate CAR. Consistent with previous results, animals treated at the earlier time point with either single-input or OR-gate CAR T cells were cleared of tumor engraftment within 6 days of T-cell injection (Fig. 3A). In contrast, all animals treated with CAR T cells on day 9 after tumor injection showed continued tumor progression for 3 days before tumor size began to
Figure 1.
Comparison of CAR T-cell preparations from multiple donors. A, T-cell subtype distribution patterns. B, exhaustion markers. Cells were stained for Tim-3, Lag-3, and PD-1. Values shown in A and B are means of three technical replicates ± 1 SD. C, viability changes during cell culture. Data shown are representative of at least two independent experiments performed on the cells from each of the three donors.

Figure 2.
Wild-type (WT) and CD19−mutant lymphoma growth in vivo after injection of OR-gate CAR or single-input CD19 CAR T cells. A, tumor progression in NSG mice bearing WT or mixed (75% WT, 25% CD19−) Raji xenografts. T cells were injected 5 days after tumor injection. B, radiance intensity of tumors engrafted in NSG mice; n = 5 in all test groups. Reported values are the means of all surviving animals in each test group, with error bars indicating ±1 SD. C, femoral bone marrow of mice bearing mixed Raji tumors and treated with mock-transduced or single-input CD19 CAR T cells analyzed by flow cytometry. Raji cells were identified by the expression of EGFP, which was stably integrated into both Raji cell lines, and CD19 staining was performed to distinguish WT and CD19−mutant Raji populations. Results represent one independent trial.
decline (Fig. 3B). Animals treated with OR-gate CAR T cells completely cleared their tumors and showed no sign of relapse at the time of this writing (through day 109). In contrast, animals treated with single-input CD19 CAR T cells experienced only temporary tumor regression before relapsing, ultimately succumbing to multifocal cancer growth (Fig. 3B). Postmortem analysis revealed tumor engraftments in the liver and the chest cavity (Fig. 3C). The recovered Raji tumors, which were identified by their luciferase and EGFP expression (Fig. 3C), were CD20+ but had either no CD19 expression or significantly reduced CD19 expression (Fig. 3D), highlighting the risk of antigen escape with single-input CD19 CAR T cells and the OR-gate CAR T cells’ ability to safeguard against this tumor defense mechanism.

Patient relapse due to loss of CD19 antigen expression has been observed in multiple clinical trials, and antigen escape has been identified as a potential pitfall of CD19-directed therapies for B-cell malignancies (3, 4). The CD19/CD20 bispecific, OR-gate CAR demonstrates the ability to address this critical medical challenge and increase the efficacy of adoptive T-cell therapy for cancer.

Published online July 1, 2016.
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doi:10.1158/2326-6066.CIR-15-0231

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