Addendum

Cancer Immunology Research

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

Eugenia Zah, Meng-Yin Lin, Anne Silva-Benedict, Michael C. Jensen, and Yvonne Y. Chen

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, 5×10^5 Raji cells were delivered by tail-vein injection and allowed to engraft prior to treatment with 10×10^6 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% $CD19^-$-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 $CD19^-$-mutant 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 $CD19^-$-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 $CD19^-$ mutants.

Mice were injected with 5×10^5 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 10×10^6 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

doi: 10.1158/2326-6066.CIR-16-0108

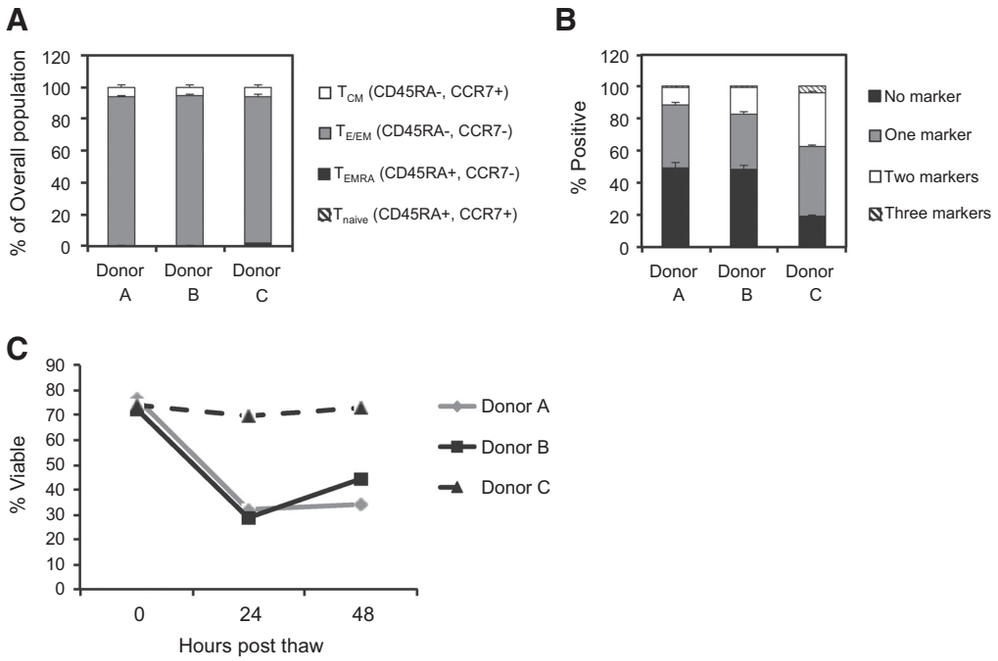

Figure 1.
Comparison of CAR T-cell preparations from multiple donors. A, T-cell subtype distribution patterns. B, exhaustion markers. Cells were stained for Tim-3, Lag-3, and PD-1. Values shown in A and B are means of three technical replicates ±1 SD. C, viability changes during cell culture. Data shown are representative of at least two independent experiments performed on the cells from each of the three donors.

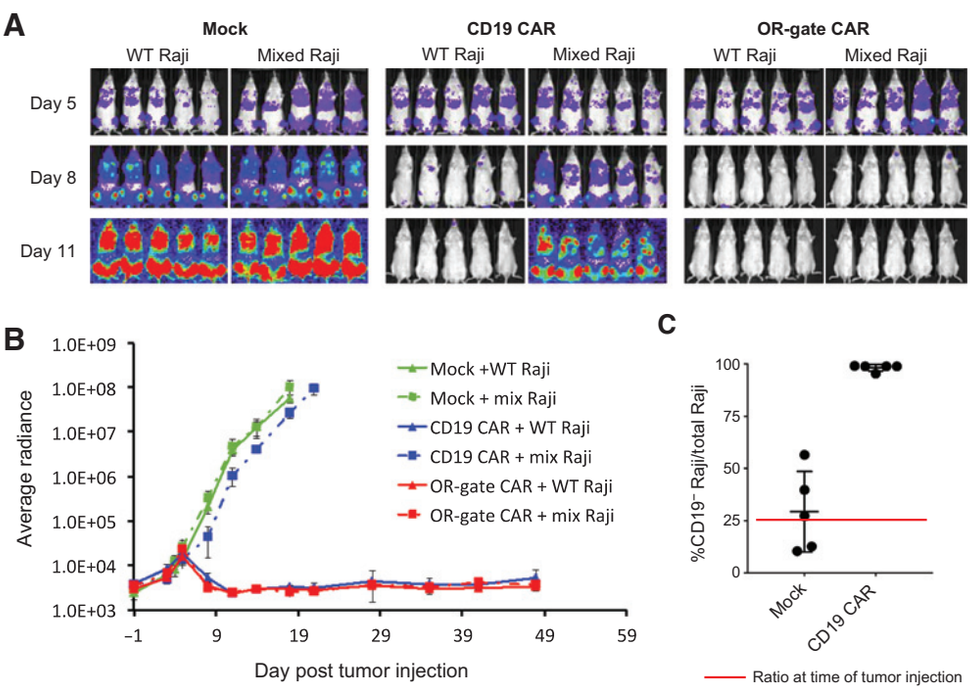

Figure 2.
Wild-type (WT) and CD19^{-}-mutant lymphoma growth *in vivo* after injection of OR-gate CAR or single-input CD19 CAR T cells. A, tumor progression in NSG mice bearing WT or mixed (75% WT, 25% CD19^{-}) Raji xenografts. T cells were injected 5 days after tumor injection. B, radiance intensity 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.

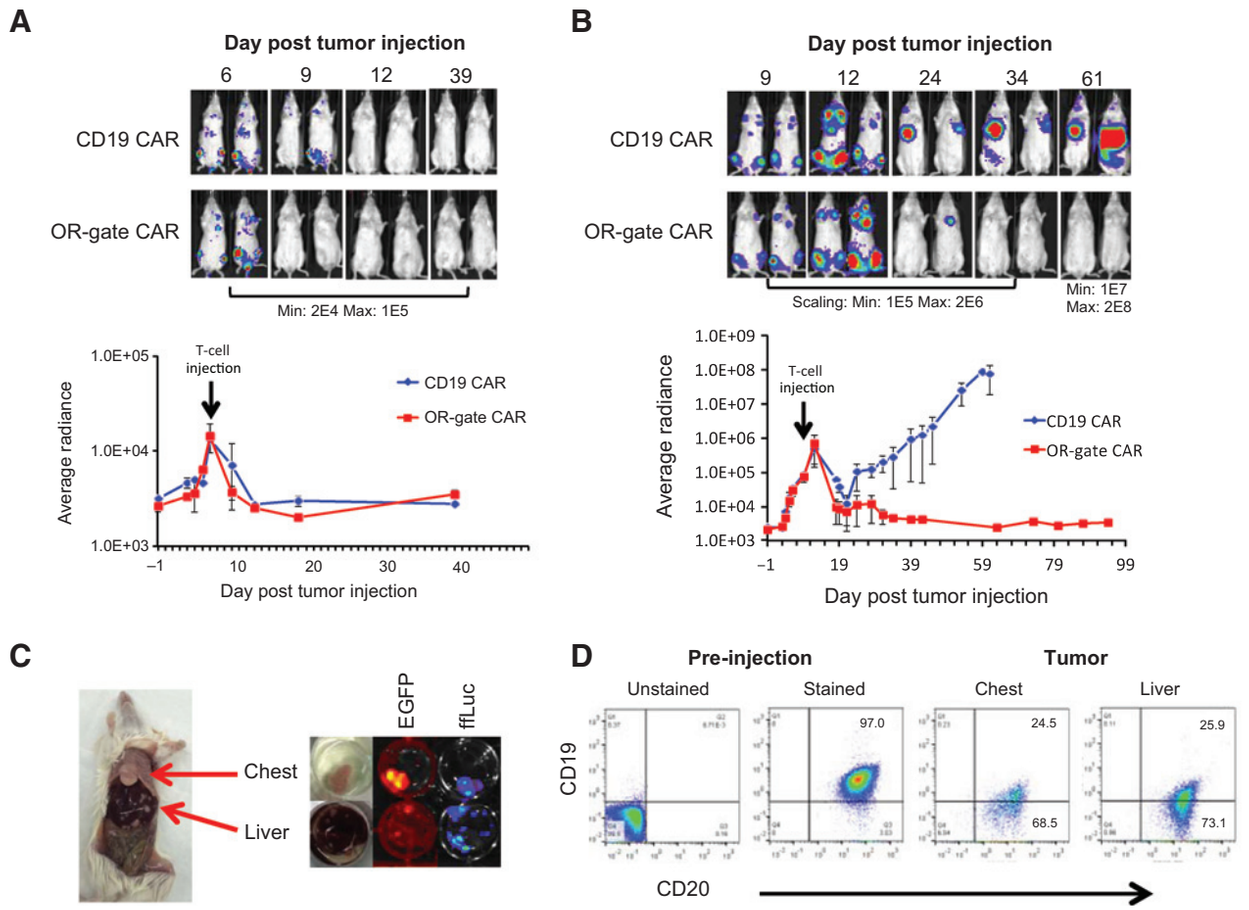

Figure 3.

Prevention of spontaneous CD19^{-}-mutant tumor outgrowth by OR-gate CAR T cells. A and B, NSG mice bearing WT Raji xenografts were treated with CAR T cells either (A) at the first sign of tumor engraftment (day 6 after tumor injection in this trial) or (B) after the tumor had expanded by 6 folds (day 9 after tumor injection). C, Raji tumor nodules were recovered from relapsed animals and their identity was verified by EGFP and firefly luciferase (ffLuc) expression via IVIS imaging. D, tumor nodule recovery from animals treated with CD19 CAR T cells. $n = 2$ in all test groups. Radiance values reported in A and B are the means of two animals with error bars indicating the data range. Signal intensity scaling in images in A and B were adjusted as indicated for visual clarity. 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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Cancer Immunol Res 2016;4:639-641.