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Multiple Injections of Electroporated Autologous T Cells Expressing a Chimeric Antigen Receptor Mediate Regression of Human Disseminated Tumor

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Abstract

Redirecting T lymphocyte antigen specificity by gene transfer can provide large numbers of tumor-reactive T lymphocytes for adoptive immunotherapy. However, safety concerns associated with viral vector production have limited clinical application of T cells expressing chimeric antigen receptors (CAR). T lymphocytes can be gene modified by RNA electroporation without integration-associated safety concerns. To establish a safe platform for adoptive immunotherapy, we first optimized the vector backbone for RNA *in vitro* transcription to achieve high-level transgene expression. CAR expression and function of RNA-electroporated T cells could be detected up to a week after electroporation. Multiple injections of RNA CAR–electroporated T cells mediated regression of large vascularized flank mesothelioma tumors in NOD/scid/γc(−/−) mice. Dramatic tumor reduction also occurred when the preexisting intraperitoneal human-derived tumors, which had been growing *in vivo* for >50 days, were treated by multiple injections of autologous human T cells electroporated with anti-mesothelin CAR mRNA. This is the first report using matched patient tumor and lymphocytes showing that autologous T cells from cancer patients can be engineered to provide an effective therapy for a disseminated tumor in a robust preclinical model. Multiple injections of RNA-engineered T cells are a novel approach for adoptive cell transfer, providing flexible platform for the treatment of cancer that may complement the use of retroviral and lentiviral engineered T cells. This approach may increase the therapeutic index of T cells engineered to express powerful activation domains without the associated safety concerns of integrating viral vectors. *Cancer Res; 70(22); 9053–61. ©2010 AACR.*

Introduction

Adoptive transfer of CTLs has shown great promise in both viral infections and cancers. After many years of disappointing results with chimeric antigen receptor (CAR) T-cell therapy, improved culture systems and cell engineering technologies are leading to CAR T cells with more potent antitumor effects (1). Results from recent clinical trials indicate improved clinical results with CARs introduced with retroviral vectors (2, 3). Perhaps not surprisingly, these CAR T cells also exhibit enhanced toxicity (4, 5). Recent editorials have discussed the need for safer CARs (6, 7).

The receptor transfer strategies described above used retroviral vector transduction that results in stable genomic integration of the transgene. This allows for constitutive expression of the transgenic receptors. However, the integration of the provirus into the genome bears the risk of insertional mutagenesis and, at least theoretically, malignant transformation of the transduced cells. In addition, stable expression of the transgene may be a disadvantage when unintended cross-reactivity of the transgenic immunoreceptor results in severe adverse effects as reported recently (5, 8).

Here, we report that by combining a robust T-cell culture system (9) with the optimized mRNA CAR electroporation protocol described herein, we have developed a platform that has the potential to increase the therapeutic window with CARs that contain increasingly potent signaling domains. Using good manufacturing practice (GMP)–grade RNA encoding a CAR against mesothelin, a glycosylphosphatidylinositol–linked molecule that is overexpressed on ovarian and pancreatic cancer and mesothelioma (10), we show robust antitumor effects in preclinical models. Most notably, significantly prolonged survival and reduced tumor burden was observed in treated mice compared with

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control groups, even when using autologous T cells from a patient with advanced metastatic cancer. Electroporation of T cells with optimized RNA CARs provides a novel and cost-efficient platform for the treatment of cancer without the associated safety concerns of integrating gene vectors.

Materials and Methods

Construction of *in vitro* transcription mRNA vectors for CARs

Mesothelin (ss1) and CD19-specific CARs (11, 12) were optimized as described in detail in Supplementary Materials and Methods.

RNA *in vitro* transcription

Three RNA *in vitro* transcription (IVT) systems were used to optimize RNA expression in T cells as described in detail in Supplementary Materials and Methods.

T-cell culture

Anonymous healthy donors donated lymphocytes at the University of Pennsylvania Apheresis Unit after informed consent under an Institutional Review Board–approved protocol, and T cells were purified by elutriation. In some experiments, we used cryopreserved T cells and tumor cells from the same patient. "Patient 108" had malignant mesothelioma. As part of an earlier clinical trial, this patient underwent leukapheresis and had tumor cells generated from his malignant pleural effusion. T cells were activated by addition of CD3/CD28 beads (Invitrogen) and a single cycle of stimulation as described (9). For the experiment shown in Fig. 5, patient 108 T cells were stimulated with irradiated artificial antigen-presenting cells expressing 4-1BBL and loaded with anti-CD3 monoclonal antibody (mAb) OKT3 and CD28 mAb 9.3 as described (13). T cells were maintained at a density of 0.8×10^6 to 1×10^6 cells/mL in RPMI 1640 with 10% FCS and 1% penicillin-streptomycin (R10) after bead stimulation.

RNA electroporation of T cells

Activated T cells were electroporated on day 10 of culture as described in Supplementary Materials and Methods.

Flow CTL

A slightly modified version of a flow cytometry cytotoxicity assay was used (14).

Mouse xenograft studies

Studies were performed as previously described with certain modifications (15, 16) as described in Supplementary Materials and Methods.

Statistical considerations

Analysis was performed with STATA version 10 (StataCorp) or Prism 4 (GraphPad Software). *In vitro* data represent means of duplicates, and comparisons of means were made via Mann-Whitney test. For comparison among multiple groups, Kruskal-Wallis analysis was performed with Dunn multiple comparison tests to compare individual groups. Survival curves were compared using the log-rank test with a Bonferroni correction for comparing multiple curves.

Results

Electroporation of RNA CARs mediates variable expression in stimulated T cells

We have previously reported that anti-mesothelin ss1 scFv CARs with combinations of CD3ζ, CD28, and 4-1BB activation domains are highly and stably expressed in T cells when introduced using lentiviral vector technology (11). Human T cells were activated for 10 days as previously described (9), and as the cells returned to a near resting state, they were electroporated with RNA encoding the ss1 scFv with the previously described combinations of signaling moieties. We found that the level of transgene expression was not uniform (Supplementary Fig. S1), as T cells electroporated with CAR bearing CD3ζ alone (ss1-z) showed the highest transgene expression, followed by nearly equivalent levels of ss1-28z (CD28 + CD3ζ) and ss1-bbz (4-1BB + CD3ζ) expression. Because "second-generation" CARs containing costimulation domains seem superior in several preclinical and early-stage clinical trials when expressed with viral vector systems (11, 12, 17, 18), we decided not to optimize expression of the "first-generation" ss1-z CAR. Rather, the second-generation ss1-bbz and CD19-bbz CARs were chosen for further optimization using RNA electroporation because they are being tested in a clinical trial using lentiviral vector technology (Clinicaltrials.gov NCT00891215).

Optimization of RNA constructs improves transgene expression in stimulated T cells

Structural modification of noncoding regions by incorporation of two repeats of 3′ untranslated regions (UTR) from β-globulin and longer poly(A) sequences has been shown to enhance RNA stability, translational efficiency, and the function of RNA-transfected dendritic cells (19). However, these strategies have not been systematically evaluated in RNA-electroporated T cells. To test if this approach applies to human T lymphocytes, we modified our IVT vector (pGEM-ss1bbz.64A) by adding 5′UTR (SP163) or 3′UTR [two repeats of 3′UTR derived from human β-globin (2bgUTR) or a prolonged poly(A) (150A) sequence as shown in Fig. 1A]. The SP163 translational enhancer is derived from the 5′UTR of the vascular endothelial growth factor gene and is reported to increase expression levels 2- to 5-fold compared with promoter alone (20). RNA made from these constructs was electroporated into stimulated T cells. As shown in Fig. 1B, compared with our basic IVT construct containing a 64-poly(A) tract, addition of 3′UTR from β-globulin (2bgUTR) and longer poly(A) (150A) tailing enhanced the transgene expression, especially when combined (2bgUTR.150A). In contrast, incorporation of the SP163 sequence at the 5′ end of ss1-bbz repressed transgene expression, which might be due to reduced capping efficiency when the SP163 sequence was added.

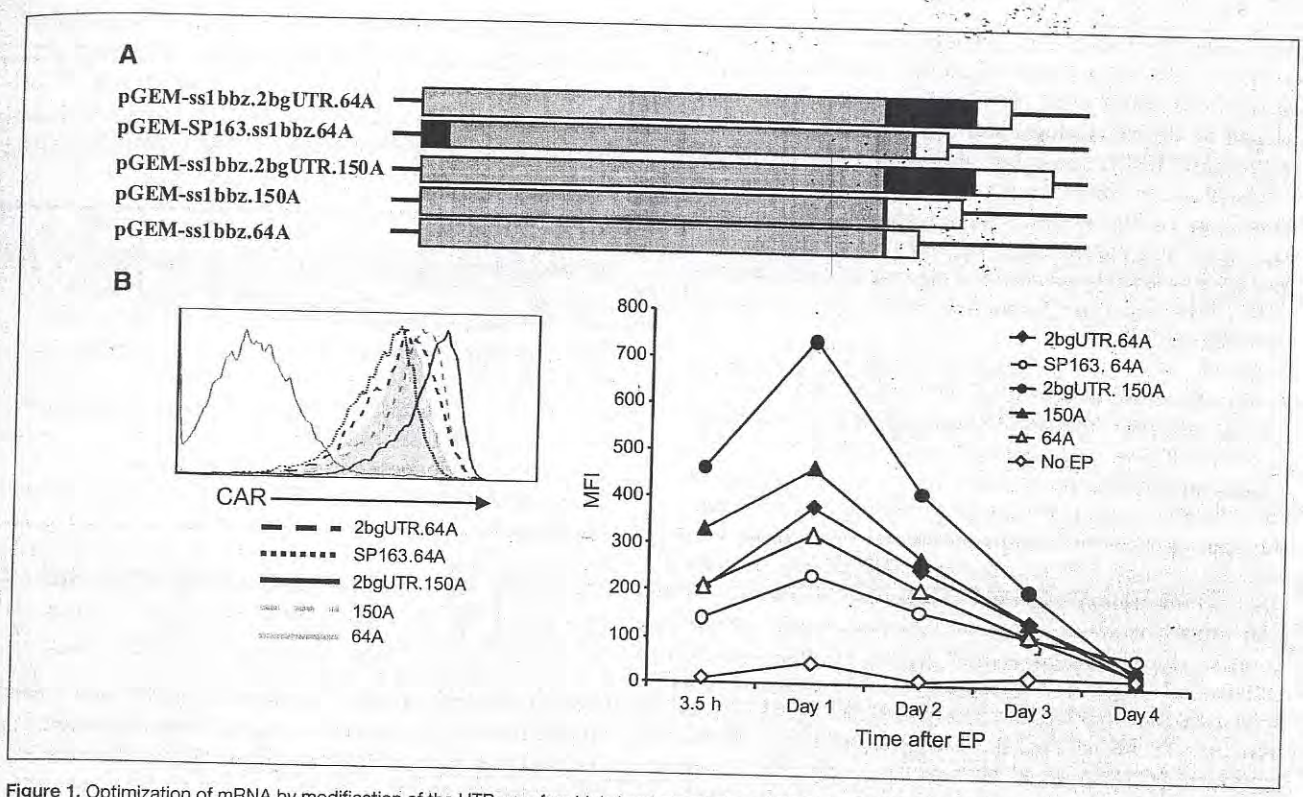

Figure 1. Optimization of mRNA by modification of the UTRs confers high-level expression of CARs in electroporated T cells. A, a schematic representation of ss1-bbz construct with different modifications of 5′UTR or 3′UTR. pGEM-based IVT vector containing ss1-bbz (pGEM-ss1bbz.64A) was modified as described in Materials and Methods to add a 3′UTR (2bgUTR.64A), a 5′UTR (SP163.64A), a longer poly(A) tail (150A), or both 3′UTR and longer poly(A) (2bgUTR.150A). B, RNA made from the modified constructs was electroporated into T cells and the transgene expression was followed by flow cytometry. Left, histograms of the transgene expression at day 1 after electroporation; right, mean fluorescence intensity (MFI) of the CAR for 4 d after electroporation. Experiments are representative of at least two independent experiments.

Optimization of the 5′ cap structure enhances the expression and function of CARs in electroporated T cells

The 5′ cap located at the end of mRNA molecule consists of a guanine nucleotide connected to the mRNA via a 5′ to 5′ triphosphate linkage. Several cap structures have been described, including caps 0 and 1 (21). Several methods have been used to incorporate the 5′ cap structure onto the transgene and poly(A) tail construct. Commercially available systems incorporate cap 0 or 1 using cotranscriptional or enzymatic approaches to produce capped mRNA. This process is important to optimize to enhance translational efficiency and because of the considerable expense of the various capping systems (see Supplementary Materials and Methods). RNA made using the different capping systems was electroporated into stimulated T cells, and the transgene expression was monitored by flow cytometry (Fig. 2A and B). The results showed that the transgene expression of T cells electroporated with RNA capped by anti-reverse cap analogue (ARCA) was 3-fold higher than regular cap (RC) analogue capped RNA at 4 hours. The transgene persistence of ARCA capped RNA was also improved, as at day 5 after electroporation >50% of the T cells still expressed the CAR as shown in Fig. 2B.

We next compared enzymatic addition of caps 0 and 1 to nonenzymatic addition of the ARCA. The potential advantage of using the capping enzyme (CE) system is that this approach includes CE and mScript 2′-*O*-methyltransferase that work together to produce the cap1 structure, which is very similar to ARCA and provides superior translation efficiency in many *in vivo* systems. To evaluate the efficiency of cap 0 or 1 RNA encoding ss1-bbz, human T cells were electroporated with RNA made by ARCA, CE, cap1 CEs, or CEs plus additional poly(A). As shown in Fig. 2C, the CAR expression using cap1 RNA electroporation was equivalent to ARCA IVT mRNA. The transgene expression was further enhanced by incorporation of the longer poly(A) tail, consistent with the results in Fig. 1.

One potential functional advantage of optimized IVT RNA is that CAR expression could be sustained, as translation of additional CAR could lead to more persistent expression and overcome downregulation induced by target recognition or homeostatic expansion. Activated T cells were electroporated with various RNA preparations encoding ss1-bbz, and then cocultured with K562-meso or control K562-CD19 target cells for 2 days (Supplementary Fig. S2). T cells electroporated with ARCA and CE1 or CE1+A capped ss1-bbz RNA could still maintain their transgene expression

after being stimulated with the K562-meso cell line compared with the same T cells cocultured with control target cells. In contrast, T cells electroporated with ss1-bbz RNA capped by the RC analogue did not have detectable CAR on the surface after cocultured with antigen-bearing target.

Based on the above results and other data (data not shown), we concluded that RNA capped with ARCA or with cap1 and a long poly(A) tail is the best RNA production system among the RNAs tested. For large-scale GMP production of IVT RNA, when the production cost is also considered, cap1 is preferred.

In vitro function of optimized IVT RNA CARs

RNAs prepared from both plasmids bearing parental or internal ORF-free CAR sequences were electroporated into T cells, and it was found that the transgene expression from the RNAs with internal ORF-free electroporated T cells was equivalent to the T cells electroporated with RNAs with parental sequences (Supplementary Fig. S3) at 20 hours after electroporation. However, substantial prolongation of CAR expression was observed in activated T cells electroporated with clinical-grade RNA generated from internal ORF-free pD-A.ss1.OF or pD-A.19.OF RNAs using the CE system that incorporated both cap1 and prolonged poly(A) into the IVT RNAs (Fig. 3). Transgene expression of the optimized IVT RNA could be detected as long as 7 days after RNA electroporation for both meso and CD19 RNA CARs as shown in Fig. 3C.

Previous studies have shown that 4-1BB is upregulated on $CD8^+$ T cells after T-cell receptor stimulation (22). We incubated bulk T cells electroporated with ss1-bbz or CD19-bbz RNA with target cells expressing either mesothelin or CD19, and found robust upregulation of 4-1BB, particularly on $CD8^+$ T cells, which was target specific (Fig. 3A). The T cells expressing RNA CARs also secreted substantial amounts of interleukin-2 (IL-2) and translocated CD107a on target-specific recognition (Fig. 3B and D). Finally, in a flow-based lytic assay, we found that both CD19 (19.OF) and ss1 (ss1.OF) CAR RNA–electroporated T cells could specifically lyse target cells efficiently (Supplementary Fig. S4).

RNA-electroporated T cells mediate regression of human disseminated mesothelioma xenografts

A pilot experiment was first conducted to evaluate the therapeutic potential of T cells expressing optimized RNA CARs in mice bearing large pre-established tumors. Mesothelin-positive tumors were established in NSG mice as previously reported (11). Sixty-six days after tumor inoculation, 10×10^6 to 15×10^6 ss1-bbz RNA CAR–electroporated T cells from a healthy donor were injected intratumorally, twice weekly for 2 weeks. The biweekly administration schedule was based on the *in vitro* expression data shown in Fig. 3. As seen in Fig. 4A, the tumors regressed in the mice treated with ss1 RNA–electroporated T cells, whereas progressive tumor growth was observed in the control group of mice. At the time the mice were sacrificed on day 98, tumor size was substantially smaller in all of the mice treated with electroporated T cells than that of the mice treated with saline (Supplementary Fig. S5). These results indicate therapeutic potential of multiple injections of RNA CAR T cells; however, they are not as potent in the same tumor model using lentiviral transduced T cells, where two intratumoral injections of T cells were able to cure most mice (11).

We developed the M108-Luc model to test if RNA CAR–electroporated T cells are capable of treating mice bearing large disseminated tumors. M108 parental cells were stably transduced with firefly luciferase to allow for bioluminescence imaging (BLI), and in preliminary experiments, we

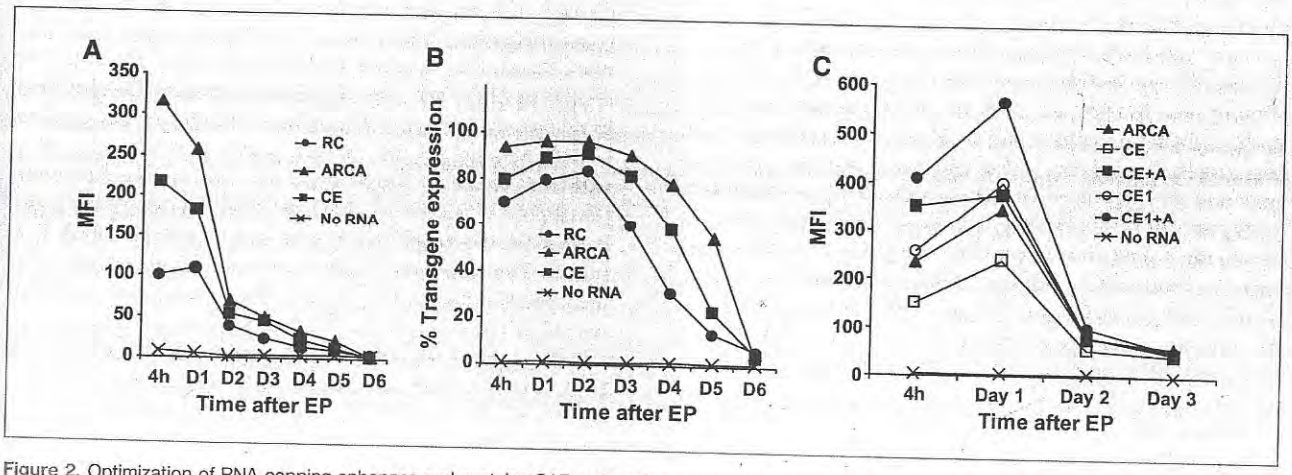

Figure 2. Optimization of RNA capping enhances and sustains CAR expression on electroporated T cells. A, T cells were electroporated with IVT RNA capped by the indicated capping method, including using RC analogue, ARCA, or CE at a fixed RNA dose of 2.5 μg/100 μL T cells. Transgene expression was monitored by measuring MFI using flow cytometry at the indicated times after electroporation (EP). B, T cells from the above experiment were monitored by flow cytometry to determine the fraction of cells expressing the transgene. C, T cells electroporated with IVT RNA encoding ss1-bbz capped by different capping methods, including ARCA, CE, CE with addition poly(A) (CE+A), CE system–generated cap1 RNA (CE1), or CE system–generated cap1 RNA plus enzymatic poly(A) (CE1+A) at an RNA dose of 10 μg RNA/100 μL T cells. Transgene expression was monitored by flow cytometry (MFI) for 3 d after electroporation. Experiments are representative of two independent experiments.

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