Cancer Research

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.

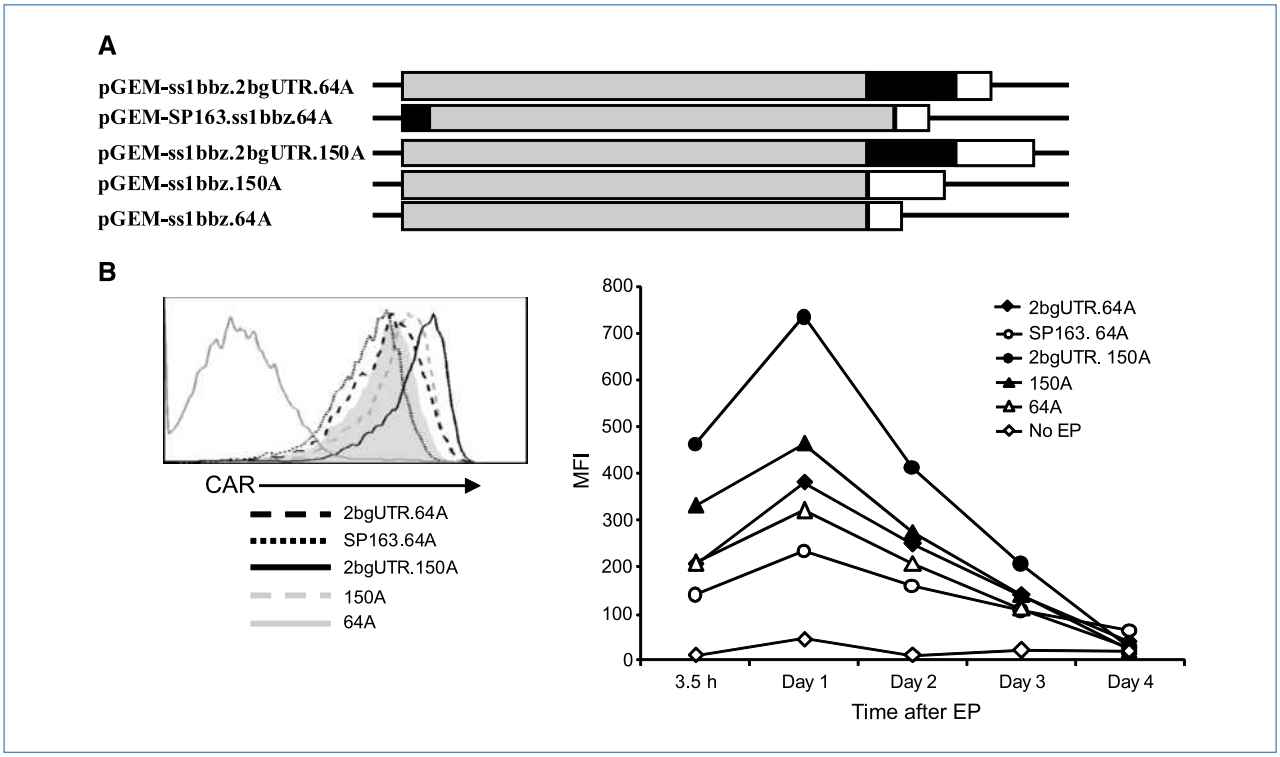

Figure 1. Optimization of mRNA by modification of the UTRs confers high-level expression of CARs in electroporated T cells. A, a schematic representation of ss1-bbz construct with different modifications of 5′UTR or 3′UTR. pGEM-based IVT vector containing ss1-bbz (pGEM-ss1bbz.64A) was modified as described 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

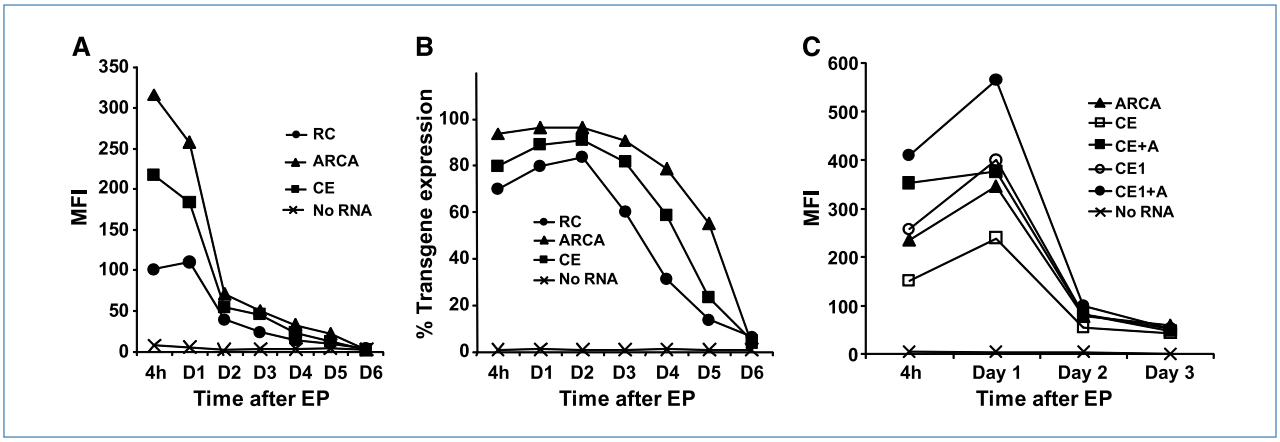

Figure 2. Optimization of RNA capping enhances and sustains CAR expression on electroporated T cells. A, T cells were electroporated with IVT RNA capped by the indicated capping method, including using RC analogue, ARCA, or CE at a fixed RNA dose of 2.5 μg/100 μL T cells. Transgene expression was monitored 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.

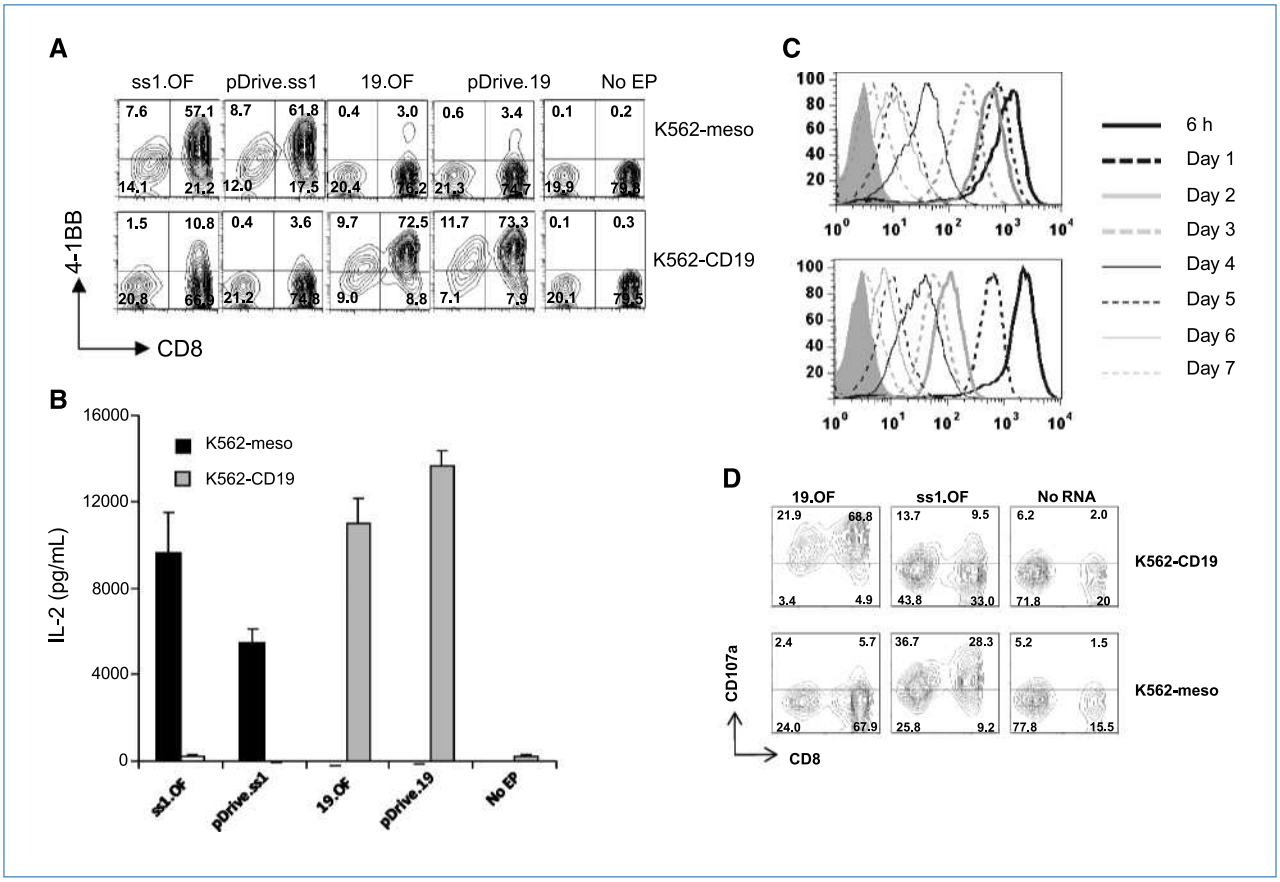

Figure 3. Sustained RNA CAR expression and function using RNA generated from regulatory-compliant vector constructs. Four hours after electroporation, the T cells electroporated with the indicated RNA were cocultured with K562-meso or K562-CD19 for 16 h. A, antigen-specific T-cell activation was detected by the induction of 4-1BB expression. B, IL-2 production was measured by ELISA. C, stimulated T cells were electroporated with clinical-grade RNA (10 μg RNA/100 μL T cells) generated from pD-A.ss1.OF (top) or pD-A.19.OF (bottom) and the transgene expression was monitored at the time as indicated. D, 1 d after electroporation, RNA CAR T-cell function was tested by measuring CD107a surface translocation after T cells expressing the indicated RNA CAR were cocultured for 4 h with K562-CD19 or K562-meso targets. The effector cells were gated on CD3. Experiments are representative of at least two independent experiments.

found that NSG mice develop widely disseminated disease with progressive ascites and that all mice die or become moribund by day 100 (data not shown). NSG mice (n = 18) were injected with M108-Luc, and they were randomized into three i.p. treatment groups. On day 58 day after tumor injection, when all mice had large vascularized tumors with ascites and metastatic nodules lining the peritoneal cavity, ss1-bbz RNA CAR–electroporated T cells from a healthy donor were injected (i.p.) into the mice, twice weekly, for 2 weeks. As a control for CAR specificity, a group of mice was injected with CD19-bbz RNA CAR T cells, and another group was treated with saline. Tumor burden in the ss1-bbz RNA CAR group progressively decreased from baseline on day 53. Furthermore, on day 78 after tumor inoculation, the tumor growth in the ss1-bbz RNA CAR T-cell–treated group was significantly repressed ($P < 0.01$) compared with both saline or CD19-bbz RNA T-cell–treated groups (Fig. 4B). In a side by side experiment, a mouse treated with ss1-bbz CAR T cells expressed using a lentiviral vector exhibited a more robust treatment effect (Fig. 4C), similar to our previously published data (11). However, the ss1-bbz RNA CAR T-cell–treated group had a survival advantage and a significant slowing of tumor growth between days 72 and 92, at which point all of the control mice died from tumor progression (Fig. 4C).

RNA CAR–electroporated autologous T cells mediate regression of disseminated mesothelioma

The above studies indicate that biweekly injections of RNA CAR T cells can control advanced flank and i.p. tumors, and that the inhibition is dependent on the CAR specificity, as T cells expressing the CD19 RNA CAR were not effective. However, the T cells in those experiments were obtained from healthy donors and were allogeneic to the tumor. Because allogeneic antitumor effects were observed with repeated long-term administration of RNA CAR T cells (data not shown), autologous peripheral blood mononuclear cells from the patient from whom the M108 tumor was derived were used. T cells were stimulated and electroporated using GMP grade RNA. Thirty NSG mice were randomized into

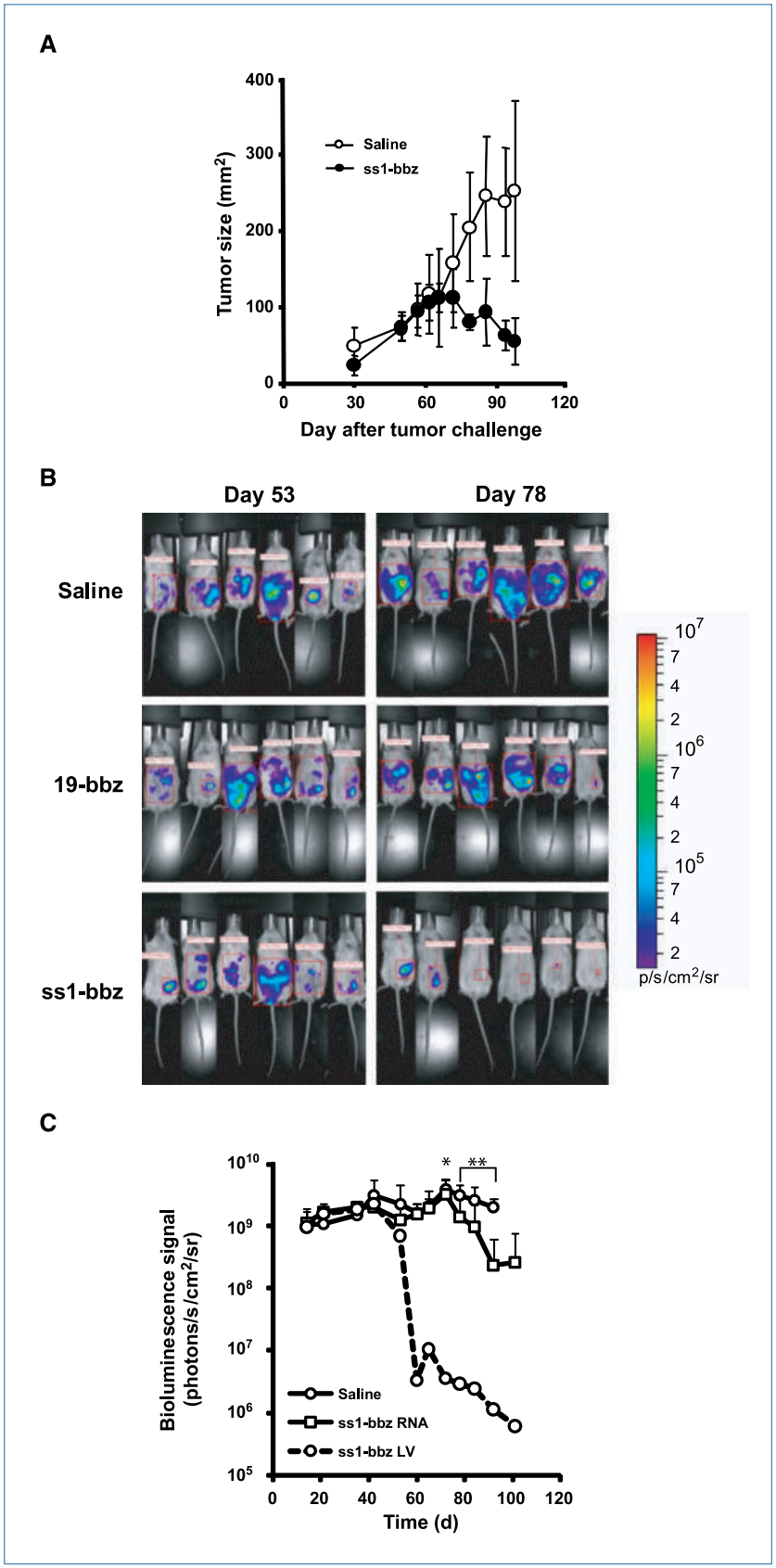

Figure 4. Regression of advanced vascularized tumors in mice treated with RNA CAR T cells. A, flank tumors were established by M108 injection (s.c.) in NOD/scid/γc(–/–) (NSG) mice (n = 6). Sixty-six days after tumor inoculation, mice were randomized to equalize tumor burden and treated with ss1-bbz RNA–electroporated T cells. The T cells (10×10^6 to 15×10^6) were injected intratumorally every 4 d for a total of four injections using the same healthy donor; mice treated with saline served as controls (n = 3). Tumor size was measured weekly. B, disseminated i.p. tumors were established in NSG mice (n = 6 per group) by i.p. injection with 8×10^6 M108-Luc cells. Beginning on day 58, RNA CAR–electroporated T cells (1×10^7) expressing ss1-bbz were injected twice weekly for 2 wk. RNA CAR T cells expressing CD19-bbz RNA CAR or saline were injected as controls. On day 78, the luminescence signal was significantly decreased in the ss1-bbz mice compared with the CD19-bbz mice ($P < 0.01$). C, BLI from a single mouse treated with a single injection on day 58 of T cells (1×10^7) expressing the ss1-bbz CAR using a lentiviral vector. BLI data for the experiment described in B are plotted. Bars, SE. *, $P < 0.05$; **, $P < 0.01$. The BLI signal in the saline group is truncated at the high end due to saturation of the imaging system.

three i.p. treatment groups, as depicted in the diagram in Supplementary Fig. S6. Mice were inoculated with M108-Luc (i.p.) on day 0 and treated with ss1-bbz or CD19-bbz RNA CAR T cells or with saline control, and tumor burden was monitored by serial BLI and body weight as indicated. Therapy was started on day 56 when the tumor was advanced based on the finding of ascites on physical examination and high BLI signals. Tumor burden was dramatically reduced in the group treated with T cells electroporated with ss1-bbz RNA CAR T cells, whereas the tumor continued to grow in the control mice treated with either CD19-bbz RNA CAR T cells or saline (Fig. 5A and C). Even in this setting, where the T cells are autologous to the tumor, there was still a modest CD19 CAR treatment effect, which may be due to the RNA backbone, as this is unlikely to be related to the CD19 scFv CAR given that there were no B cells in these mice. However, after the first six doses of T cells, imaging revealed a lower mean change tumor bioluminescence in the ss1 CAR mice (39%) compared with both the CD19 CAR (244%) and the saline mice (237%; $P < 0.001$). The 50% median survival after T-cell injection was significantly ($P < 0.05$) greater in the ss1 CAR mice (73 days) compared with the CD19 CAR (62 days) and saline mice (36 days; Fig. 5B). After the first six doses were given, the mean change in total body weight was lower in the ss1 RNA CAR mice (1.62 g) compared with both the CD19 CAR (6.21 g) and the saline mice (11.4 g; $P < 0.001$; Fig. 5C). Although we observed disease stability and even "cures" by imaging in some of the ss1 CAR mice, tumor eventually recurred. Despite giving an additional eight doses of treatment, tumor progression was observed in the ss1 CAR mice. Thus, repeated injections of ss1-bbz RNA CAR T cells can provide a survival benefit for advanced disseminated tumors. The mechanism for tumor recurrence in spite of continued therapy is under investigation.

Discussion

The goal of these experiments was to determine the therapeutic potential of activated T cells expressing electroporated RNA CARs. The main point of our article is that mRNA CARs provide a platform that is expected to be safer and more economical than retroviral or lentiviral vectors for

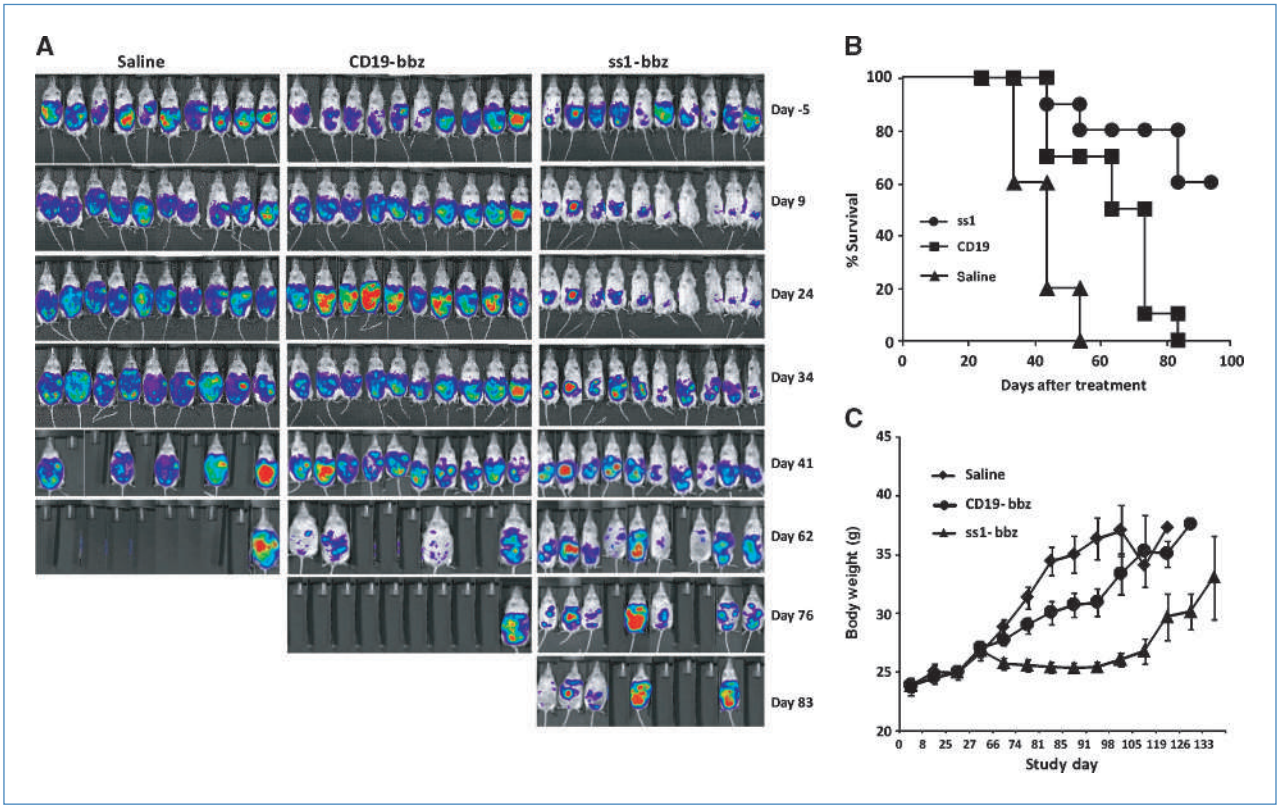

Figure 5. Multiple injections of autologous RNA CAR T cells control the growth of advanced disseminated cancer in a xenogeneic mouse model. A, NOD/scid/γc(–/–) mice ($n = 30$) were injected with 8×10^6 M108-Luc tumor cells (i.p.) and the mice were randomized into three groups before beginning therapy with RNA-electroporated autologous T cells (10^7 per injection) expressing ss1-bbz CAR, control CD19-bbz CAR, or saline on day 56 after tumor inoculation. Autologous T cells were injected i.p. and images were performed on surviving animals as indicated. Imaging commenced 5 d before the start of T-cell treatment. Tumor BLI significantly decreased in the ss1 CAR mice (38.6%) compared with both the CD19 CAR (243.6%) and the saline mice (237.1%) after the first six doses ($P < 0.001$). B, Kaplan-Meier analysis. Median survival was significantly greater in the ss1 CAR mice compared with the CD19 CAR and saline mice ($P < 0.05$). C, significantly less ascites accumulated in the ss1 CAR mice, as the mean change in total body weight was lower compared with both the CD19 CAR and the saline groups of mice ($P < 0.001$).

the evaluation of new targets. In the event of toxicity, injections of RNA CAR T cells can be terminated, and the toxicity would be expected to rapidly abate. However, the RNA CAR T cells have a substantial treatment potential, especially in compartmentalized tumors such as mesothelioma. RNA CAR T cells are expected to complement therapies currently being developed with retroviral and lentiviral CARs.

Our approach was to first optimize RNA expression and then test a multiple dosing strategy in robust tumor models. This is the first report indicating that retargeted T cells can have potent *in vivo* antitumor effects without the use of an integrating vector system. Using optimized IVT mRNA, we show that RNA CAR T cells have potent antitumor effects on advanced flank and intraperitoneal tumors. Further, as far as we are aware, our studies are the first to show that autologous T cells obtained from a patient with advanced cancer can be engineered and shown to control metastatic tumor in robust preclinical models.

We and others have previously shown that RNA electroporation can modify T-cell function *in vitro* (23 24 25). Mitchell and colleagues (26) reported that T cells can be functionally modified by RNA transfection of the chemokine receptor CXCR2 to migrate efficiently toward a variety of CXCR2-specific chemokines *in vitro* and *in vivo*. Yoon and colleagues (27) recently showed that adoptive transfer of Her2/neu RNA CAR–electroporated T cells in the SKOV3 xenograft model led to a decreased rate of tumor growth compared with transfer of mock-transfected T cells. A recent report showed the feasibility of mRNA transfection of a CD19 chimeric receptor into a natural killer cell line, but without a preclinical model or demonstration of *in vivo* effect (28). Our studies are the first to report the *in vivo* regression of large advanced tumors and survival extension using RNA-electroporated T cells.

There are a variety of nonintegrating approaches to engineer T cells (29). A temporary expression approach toward CAR immunotherapy, such as mRNA transfection, runs counter to our previous efforts and to those of most investigators in the field. However, the improving technology for RNA transfection may compliment the use of CARs that are stably expressed by integrating viral vector or transposon systems. By systematic comparison of 3′UTR and 5′UTR, incorporation of longer poly(A) tails, efficient capping of RNA, and removal of internal ORFs we were able to achieve high-level and longer expression of RNA CARs in electroporated T cells.

The prevailing paradigm in the adoptive transfer field is that long-term persistence of the cells within the patient is key to efficacy (30, 31). However, it is being increasingly realized that transferred cells can lose their ability to function within the tumor microenvironment rather quickly (32). Our data suggest that it may be possible to give multiple, more frequent injections of T cells that only temporarily express the transgenes of choice, avoiding the accumulation of CAR T cells that have become tolerized, and therefore achieve antitumor efficacy with an improved safety profile. Alternatively, the improving technology for RNA transfection may complement the use of CARs that are stably expressed by integrating viral vector or transposon systems.

Several adverse events have been observed and others are theoretically possible with CAR T-cell therapy. Two deaths have recently been reported following treatment with retrovirally modified CAR T cells, and the early toxic events have been related to systemic effects from cytokine release (4, 5). As a consequence of these clinical events, recent editorials have discussed the need for safer CARs (6, 7). Other toxicities encountered with stably transduced CAR T cells have been on-target, off-organ effects such as the depletion of normal B cells following CD19 CAR therapy, or the induction of hepatic toxicity following carbonic anhydrase IX therapy (8). Although not tested in this work, it is likely that repeated administration of RNA CARs would be required to elicit this form of toxicity, and that the toxicity would resolve following discontinuation of RNA CAR T-cell infusions. Finally, concerns over the lentiviral or retroviral introduction of CARs into CTLs include the known risk of malignant transformation from insertional mutagenesis (33, 34). As there is no integration into the host cell genome and the CAR expression is self-limited, these concerns are circumvented by mRNA transfection.

The primary potential limitation of CAR therapy is the relatively short persistence of RNA CARs. This can be expected to be exacerbated when the RNA CAR T cells are administered to hosts that have been lymphodepleted, which would be expected to result in the induction of homeostatic proliferation of the CAR T cells and, as a consequence, the accelerated loss of CAR expression at the T-cell surface. Thus, RNA CAR T cells may be more effective when given for compartmentalized tumors such as mesothelioma or central nervous system tumors. Furthermore, more frequent administration of RNA CARs may be required in lymphodepleted hosts.

In addition to providing a form of toxicity management discussed above, there are several potential opportunities for RNA CAR T-cell therapy. First, RNA CARs offer the potential to accelerate the pace of CAR development, by providing a flexible and more rapid path to the clinic, and thereby enabling an efficient iterative approach to optimize CAR design and potency. Based on the data in these studies, we plan to open a phase I trial testing anti-mesothelin RNA CARs. The regulatory approval process is less cumbersome with RNA CARs than with stably expressed CARs that require genomic integration. Clinical-grade mRNA is less costly to produce than integrating retroviral or lentiviral vectors, although more expensive than plasmid DNA that is being used in transfection or transposon-based protocols (2, 35). Second, it may be attractive to combine RNA CAR "knockdown" therapy using potent but potentially toxic CARs for remission induction, with consolidation and maintenance therapy using stably expressed CARs as a strategy to provide CAR cells with a potential for memory.

In summary, multiple injections of RNA-engineered T cells are a novel approach for adoptive cell transfer, providing a cost-efficient and flexible platform for the treatment of cancer diseases. In addition, 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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