Anticancer effects of a single intramuscular dose of a minicircle DNA vector expressing anti-CD3/CD20 in a xenograft mouse model

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Bispecific antibodies (BsAbs) are a class of promising anticancer immunotherapies. Among them, the US Food and Drug Administration (FDA)-approved blinatumomab (BLI) is very effective in eliminating the minimum residual disease (MRD) of acute lymphoblastic leukemia (ALL), resulting in long-term remission in many individuals. However, the need for months-long intravenous delivery and high cost limit its clinical acceptance. Here we demonstrate that these problems can be solved by a BsAb expressed by one intramuscular (i.m.) dose of a minicircle DNA vector (MC). In a human B lymphoma xenograft mouse model, when microcancers became detectable in bone marrow, the mice received an i.m. dose of the MC encoding the BsAb anti-CD3/CD20 (BsAb.CD20), followed by 8 subsequent intravenous (i.v.) doses, one every other day (q2d), of human T cells to serve as effectors. The treatment resulted in persistent expression of a therapeutic level of serum BsAb.CD20 and complete regression or growth retardation of the cancers in the mice. These results suggest that the i.m. MC technology can eliminate the physical and financial burdens of i.v. delivered BLI without compromising anticancer efficacy and that cancer can be treated as easily as injecting a vaccine. This, together with other superior MC features, such as safety and affordability, suggests that the i.m. MC BsAb technology has great clinical application potential.

INTRODUCTION

Bispecific antibodies (BsAbs) are emerging as a class of promising anticancer therapies; many clinical[1] and preclinical studies[2] have demonstrated high efficacy. Three BsAbs, blinatumomab (BLI; anti-CD3/CD19) and mosunetuzumab and RNGN1979 (both anti-CD3/CD20), are already approved by the US Food and Drug Administration (FDA) for treating human hematopoietic malignancies,[3] and more than 60 additional BsAbs are currently in preclinical and clinical development.[4] Another BsAb, catumaxomab, was approved for treating malignant ascites but was withdrawn from the market in 2017. BsAbs work by directing T cells to kill cancer cells via a cytolytic immune synapse formed between the two cell types.[5] Compared with anticancer cell therapies such as tumor-infiltrating lymphocytes (TILs) and chimeric antigen receptor (CAR)-modified T cells (CAR-T cells), which need time-consuming *in vitro* expansion (and CAR-T cells also have safety concerns because of viral genome mutation insertion), BsAbs are safer and can be made as off-the-shelf products to be delivered upon request without delay. However, BLI has a half-life of about 2 h in the circulation and requires months-long continuous intravenous infusion to maintain an effective level, which imposes a difficult physical burden on affected individuals.[6,7] This, together with high cost, limit its clinical acceptance.[8] To overcome these problems, we developed a minicircle DNA vector (MC) technology allowing the DNA vector-expressed BsAb to work as efficiently as its intravenously (i.v.) delivered counterpart.

MCs are a class of enhanced nonviral DNA vectors comprising an almost solely transgene expression cassette with excellent safety and transgene expression profiles *in vitro* and *in vivo*.[9,10] In the mouse liver, the silencing effect of plasmid backbone (pBB) DNA and the superiority of the pBB-free MC in transgene expression are well documented.[9,15] In the present study, we found that the pBB-mediated silencing effect could occur in skeletal muscle as well and that the MC can express more than 10-fold higher levels of transgene product than its plasmid DNA (pDNA) counterpart. Previously, we showed that a single dose of the MC encoding BsAb.CD20 (MC.CD20) delivered to the mouse liver via a hydrodynamic procedure can express high levels of BsAb.CD20 persistently, resulting in a significant anticancer effect in xenograft mouse models.[11] In the present study, we found that MC.CD20 delivered through intramuscular (i.m.) injection, a clinically acceptable drug administration route, was as effective. These data demonstrate that the MC-expressed BsAb.CD20 can work in place of the i.v. delivered BsAb and that cancer can be treated as easily as injecting a vaccine. These and other superior features of the MC, such as a good safety profile and affordability, suggest that the i.m. MC.BsAb technology has great clinical application potential.

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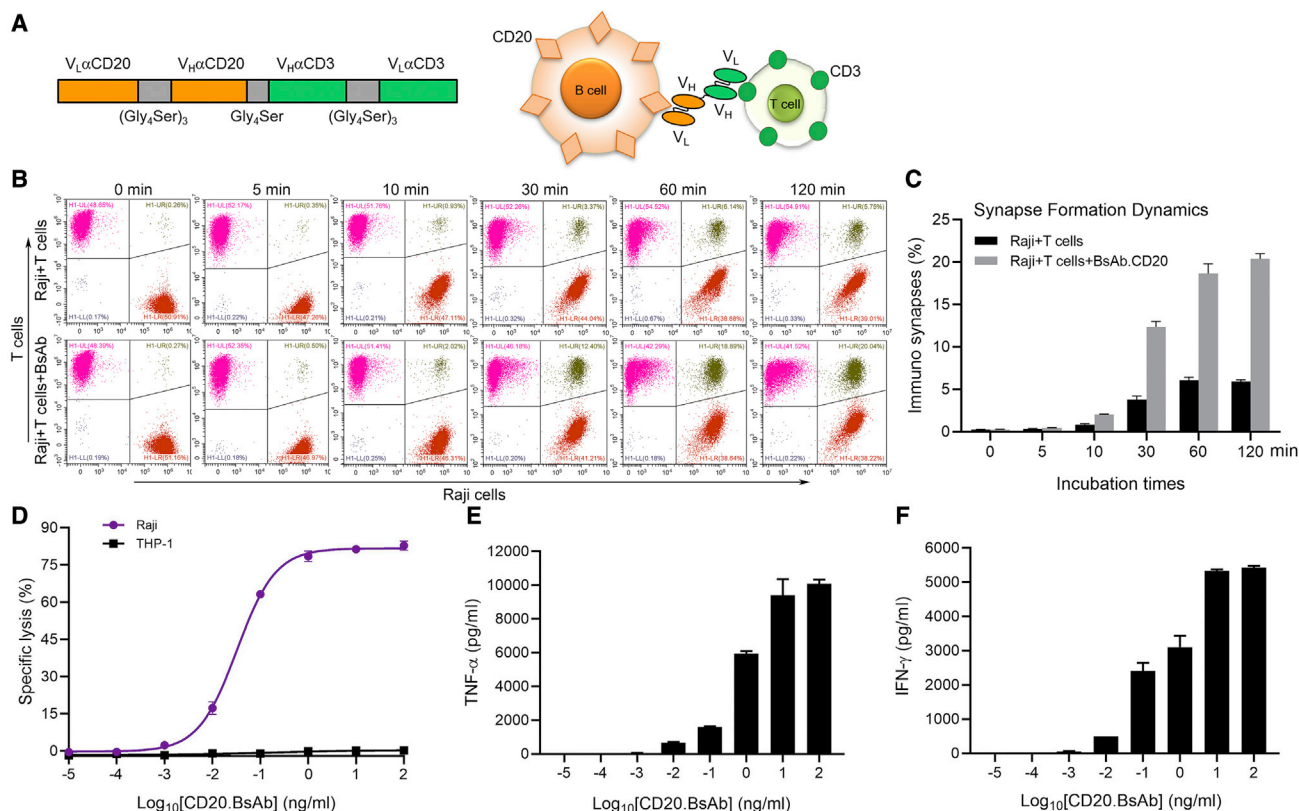**E-mail:** chenzymc@163.com

Figure 1. BsAb.CD20 construction and characterization

(A) Schematic of the structure of BsAb.CD20 in the scFv-linker-scFv format and the connection between the target B cell and effector T cell by the BsAb. Both scFvs were humanized mouse anti-human antibodies. (B) Raji and T cells were incubated with CellTrace Violet dye and CellTrace Far Red dye, respectively. BsAb-mediated immune synapse formation reactions were initiated by combining the two kinds of cells at a 1:1 ratio with (bottom row) or without (top row) the BsAb.CD20 (40 ng/mL). The frequency of immune synapse formation was measured by flow cytometry. Results of one experiment among three in the group are shown. (C) The chart illustrates the kinetics of immune synapse formation. The results are presented as mean ± SD of three independent experiments. (D) Specific lysis of Raji and THP-1 cell lines in the presence of T cells at varying doses of BsAb.CD20. (E and F) TNF-α (E) and IFN-γ (F) secretion during Raji cell killing. Each 200-μL reaction comprised 2×10^5 T cells and 5×10^4 Raji cells (E/T ratio of 4:1) with variable amount of BsAb.CD20 as indicated on the x axis. Results are from three independent experiments.

RESULTS

Construction and characterization of the MC.CD20-expressed BsAb.CD20

MC.CD20 encoding the humanized BsAb.CD20 without an Fc fragment was made according to a method established previously.[11] Briefly, the anti-CD3/CD20 expression cassette was inserted into the *Afe*I and *Sal*I sites of empty plasmid pMC.BESXP, generating the MC-producing plasmid pMC.anti-CD3/CD20. The minicircle MC.CD20 was produced using a genetically engineered *E. coli* strain ZYCY10P3S2T-based system (Figure S1). BsAb.CD20 comprised two single-chain variable fragments (scFvs). The first was the anti-CD20 scFv, which included the variable domains of the light chain (V_L) and heavy chain (V_H) of the humanized mouse anti-human CD20 monoclonal antibody (mAb) GA101[12] linked by a polypeptide $(Gly_4Ser)_3$. The second was the anti-CD3 scFv, which comprised the polypeptide $(Gly_4Ser)_3$-connected V_H and V_L domains of humanized mouse anti-human CD3 diL2K.[13] The two scFv sequences were linked by another Gly_4Ser linker, resulting in BsAb.CD20 (Figure 1A).

The BsAb.CD20-mediated CD3$^+$ T cell killing of Raji cells was triggered by formation of the cytolytic immune synapse upon binding of the two cell types simultaneously (Figure 1A). The kinetics of the immune synapse formed between the two cell types was determined to serve as the parameter of antibody strength in induction of anticancer cytotoxicity.[14] We found that, in the presence of the BsAb, the frequency of immune synapses increased with extending incubation time. However, the immune synapse was also detectable in reactions without BsAb.CD20 or CD20-negative THP-1 cells as target cells (Figures 1B, 1C, and S2). We speculated that the background values appear with increase in incubation time.

To assess the functionality of BsAb.CD20, T cells as effector cells were mixed with Raji or THP-1 cells as target cells at an effector-to-target ratio of 4:1 and incubated with 10-fold serial dilutions of BsAb.CD20 for 4 h. Dose-dependent increases in specific lysis were observed in Raji cells. The half-maximal effective concentration (EC$_{50}$) value for Raji cell lines was 0.0327 ng/mL. BsAb.CD20 had no killing activity
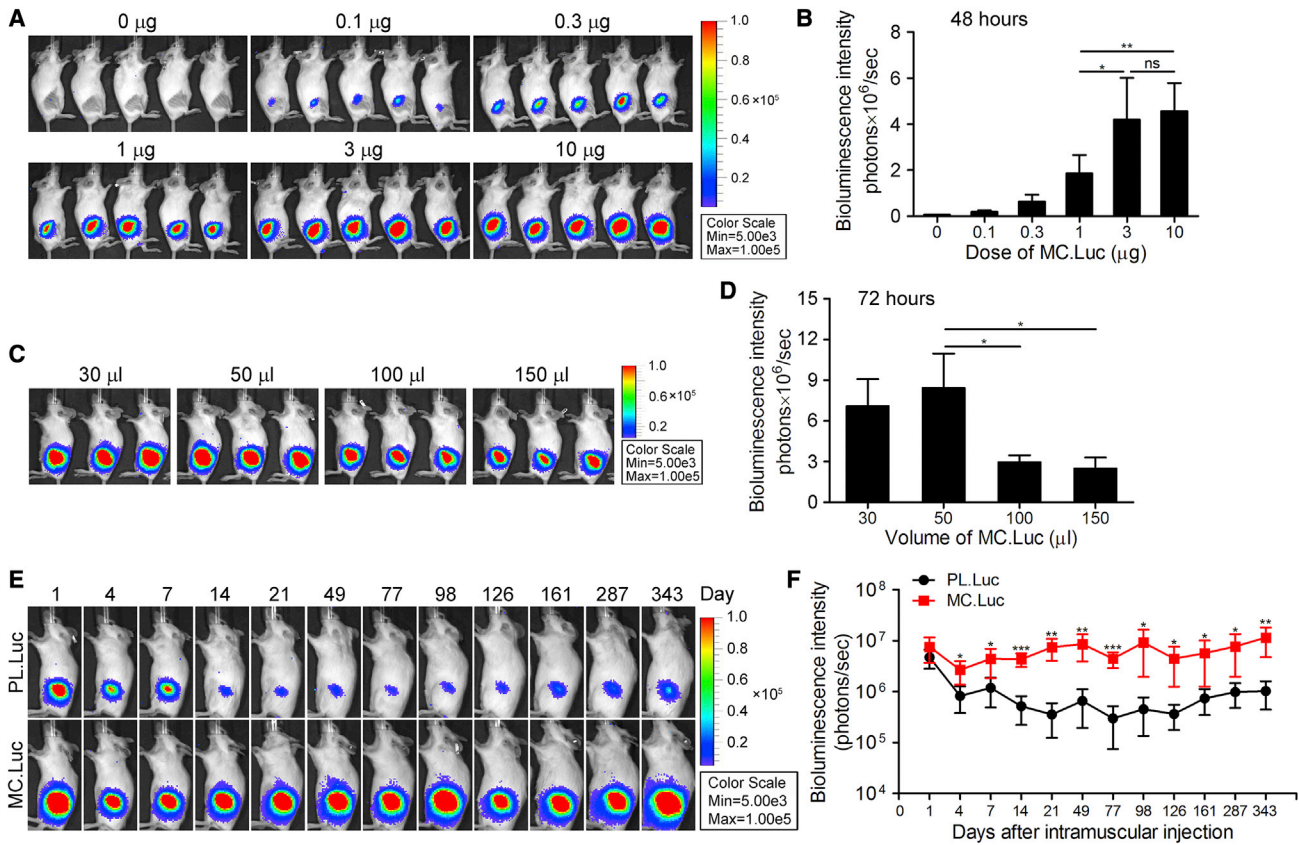
Figure 2. Optimization of the MC i.m. injection protocol

(A) To determine the optimal MC.Luc dose, groups of mice received different amounts of MC.Luc, from 0–10 µg each, and glycyrrhizin (GL) at a ratio of 4:3 in 100 µL of PBS into the left *rectus femoris* (n = 5). (B) The chart compares the bioluminescence intensity of different groups of mice in (A). (C) To determine the optimal injection volume, groups of mice received an i.m. dose of 3 µg MC.Luc and 2.25 µg GL in 30, 50, 100, and 150 µL of PBS into the left *rectus femoris* (n = 3). (D) The chart compares the bioluminescence intensity captured 72 h after DNA delivery to the mice in (C). (E) To compare the transgene expression pattern between MC and the standard plasmid (pDNA), two groups of mice received an i.m. dose of the mixtures comprising 3µg of MC.Luc or an equimolar amount of the pDNA (6 µg) in 50µL PBS (n = 5). The bioluminescence intensity was determined periodically. One mouse image in each group showed a trend of bioluminescence intensity changing with time. (F) The bioluminescence intensity of the two groups of the mice in (E) represented as a chart. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

against THP-1 cells devoid of CD20 expression (Figure 1D). As shown in Figures 1F and 1E, incubation of BsAb.CD20 with target Raji cells caused dose-dependent interferon-α(IFN-α) and tumor necrosis factor gamma (TNF-γ) release in human T cells. Consistent with the results of the killing assay, no cytokines were detected in the culture supernatant of CD20-negative THP-1 cells as target cells (data not shown).

Optimization of the MC.CD20 i.m. injection protocol

Previously, we showed that a single injection of MC.CD20 into the mouse liver via the tail vein hydrodynamic injection procedure was able to persistently express a high level of anticancer BsAb.CD20, resulting in a significant anticancer effect in a xenograft mouse model.[11] The study proved the concept that treating cancer using an MC-expressing BsAb *in vivo* is a viable technology. However, the hydrodynamic injection technique is not applicable to humans. In the present study, we explored the possibility of delivering MC.CD20 using i.m.

injection, clinically acceptable MC administration route. We conducted experiments to determine the optimal MC dose and injection volume using the minicircle expressing luciferase gene (MC.Luc) and determined that the higher transgene expression level was produced by injecting 50 µL PBS containing 3 µg of MC.Luc and 2.25 µg of glycyrrhizin (GL; weight ratio, 4:3) into each *rectus femoris* (Figures 2A–2D). GL was added because it has been shown in a pilot experiment to significantly enhance transgene expression (Figure S3). We used these optimized conditions as a common transfection procedure for i.m. delivery of MC.DNA.

Multiple earlier studies have shown that i.m. injected plasmids were able to express significant levels of transgene products, albeit for a short period of time.[15–17] It was discovered that the pBB could mediate the silencing effect to shut down expression of the transgene in mouse livers. We conducted an experiment to test whether the silencing effect occurred in skeletal muscle as well and was
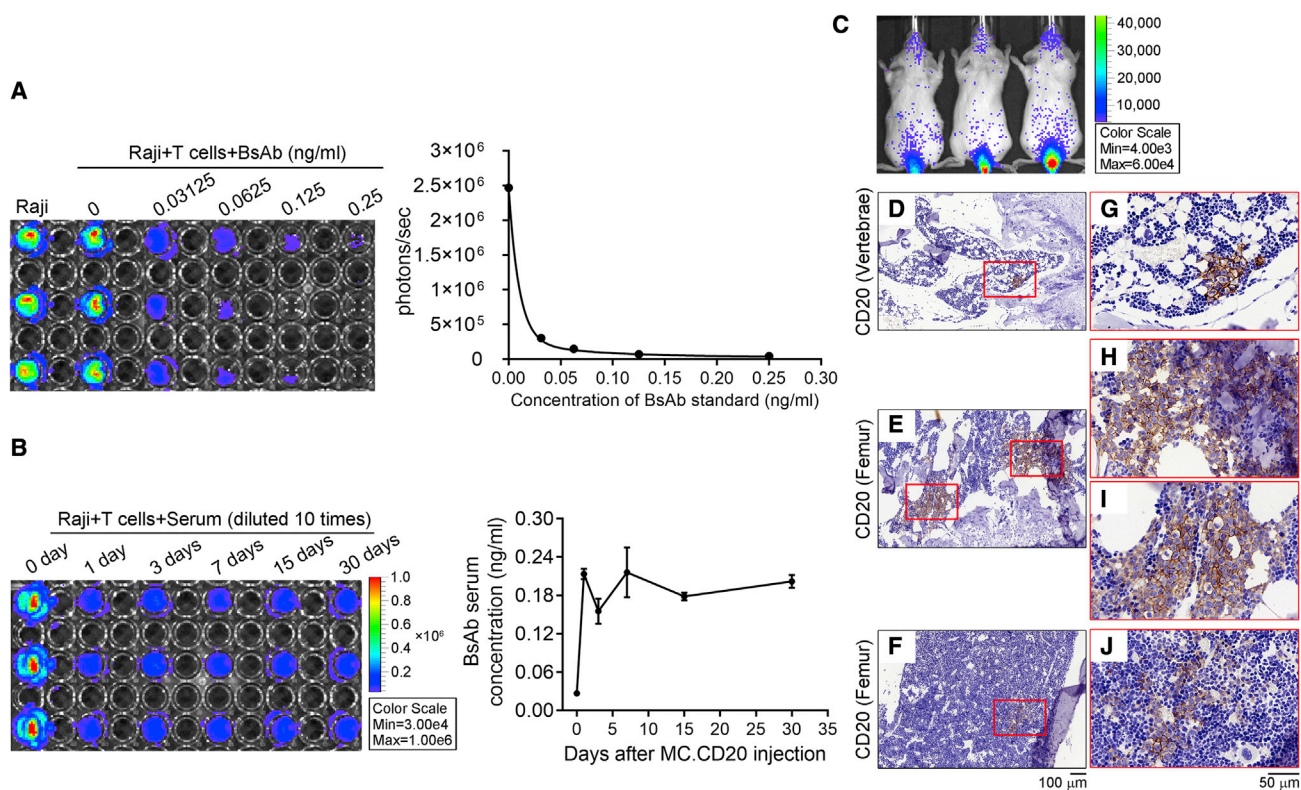
Figure 3. Determination of MC-expressed BsAb.CD20 levels in the circulation and illustration of microcancers in the bone marrow

(A) A standard curve of BsAb.CD20-mediated T cell killing of Raji cells for use with the T cell-dependent cellular cytotoxicity (TDCC) assay to determine the serum BsAb.CD20 levels in mice receiving one i.m. dose of MC.CD20. (B) The serum BsAb.CD20 levels were determined as in (A) but using different volumes of mouse serum in place of the purified BsAb.CD20. The serum was prepared from orbital blood collected from mice 1, 3, 7, 15, and 30 days after one i.m. dose of MC.CD20. (C) Three 5-week-old female NOD/SCID mice were inoculated with 5×10^5 Raji.Luc cells each through tail vein injection. Three days later, the Caliper Spectrum imaging system was used to illustrate cancer cell growth, and then the mice were killed to detect Raji.Luc cell-formed microcancers. (D–I) Immunohistology illustration of microcancers in bone marrow. Parts of vertebrae or femora from three mice were sent to Servicebio Technology for immunohistology examination of Raji cell-generated microcancers using mouse anti-human CD20 as the primary antibody. The right panel (scale bar, 100 μm) and left panel (scale bar, 50 μm) show representative images of immunohistochemistry staining.

responsible for the short expression duration demonstrated by others. Two groups of female BALB/c mice were injected i.m. with equal molar amounts of MC.Luc (3 μg MC.Luc and 2.25 μg GL) and an MC.Luc-producing plasmid (PL) counterpart (6 μg PL.Luc and 4.5 μg GL) into the *rectus femoris*. We found, consistent with what was observed in mouse livers, that both groups initially expressed high levels of luciferase, which remained stable throughout the 343-day experiment in the MC group, but dropped more than 10-fold in the pDNA group during the time (Figures 2E and 2F).

Determination of MC.CD20-expressed BsAb.CD20 levels in the circulation and establishment of a Raji cell-generated microcancer model

Based on the observation that one i.m. dose of MC.Luc could result in a persistent high level of luciferase for up to 343 days (Figures 2E and 2F), we hypothesized that one single i.m. dose of MC.CD20 could be as effective in treating B cell lymphoma as in our previous i.v. injection experiment.[11] As the first step to test this hypothesis, we determined the BsAb.CD20 expression level using the optimized MC

delivery protocol. A group of mice received one i.m. dose of MC.CD20, 3 μg/50 μL, into the *rectus femoris* of both legs, and orbital blood collected periodically was processed to determine the BsAb.CD20 expression level using a T cell-dependent cellular cytotoxicity (TDCC) assay. We found that the BsAb.CD20 level was maintained around 200 pg/mL in the serum for up to 30 days (Figures 3A and 3B).

Subsequently, we used Raji cells, a human B cell lymphoma line, to establish the microcancer mouse model as in a previous study.[11] Three female non-obese diabetic (NOD)/severe combined immunodeficiency (SCID) mice were inoculated i.v. with luciferase gene-labeled Raji (Raji.Luc) cells, 5×10^5 cells per mouse. Three days after cancer cell inoculation, although the Caliper Spectrum imaging system only detected bioluminescence spots clustering in the front and tail region in all three mice (Figure 3C), microcancers formed by Raji cells were detected in the bone marrow of all three mice, as visualized by immunohistology using mouse anti-human CD20 as the primary antibody (Figures 3D–3J). These microcancers appear to
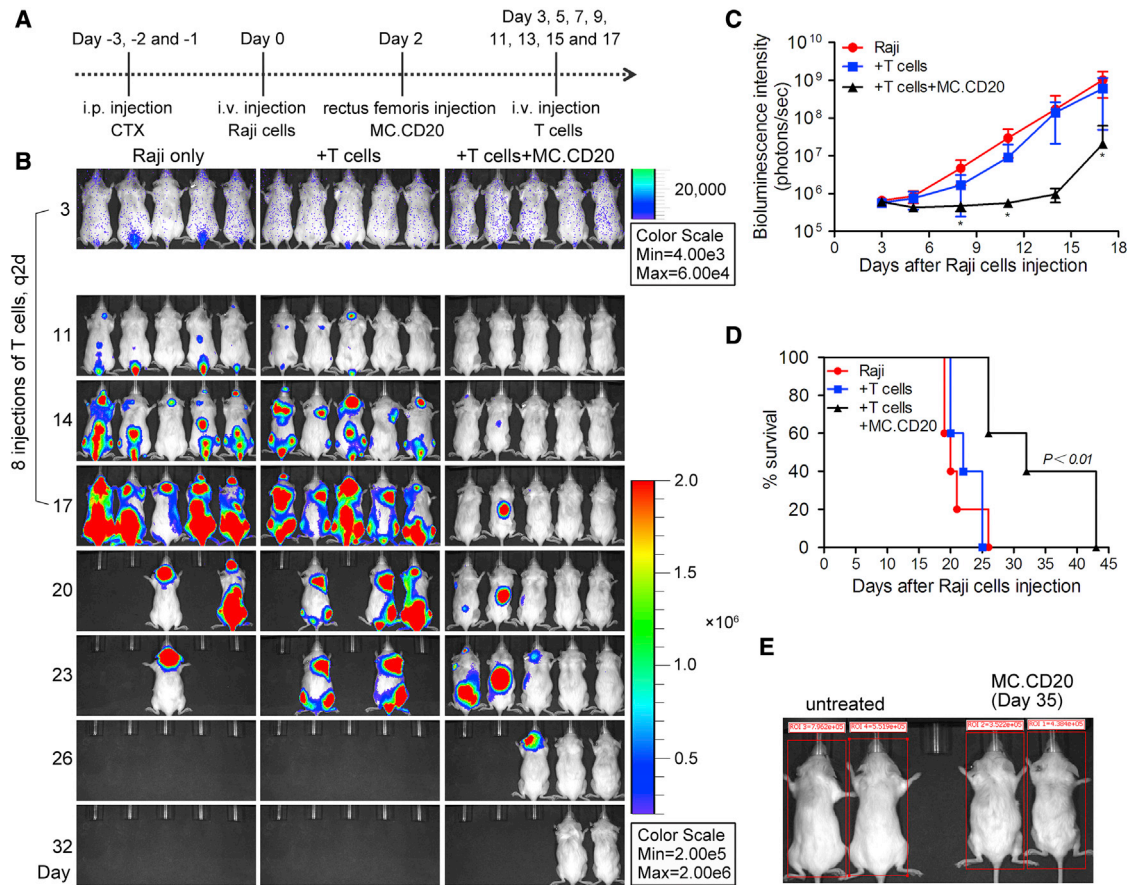
Figure 4. Therapeutic efficacy of one i.m. dose of MC.CD20

(A) Detailed schematic of the animal study. (B) Bioluminescence was captured using the Caliper Spectrum imaging system. The boxed mice at day 3 were imaged at a lower scale (n = 5). (C) The chart comparing the tumor burdens among the groups was derived from the digitalized bioluminescence intensity of individual mice in (B). An asterisk indicates that the bioluminescence intensity in the MC.CD20 group was significantly lower than in the untreated or T cell-only control group at the indicated time points. (D) The mouse survival curve was composed according to the mouse death record. The average lifespan in the MC.CD20 group was significantly longer than in the two control groups ($p < 0.01$). (E) The bioluminescence signal was examined in the two MC.CD20-treated mice killed on day 42 side-by-side with two untreated mice. There was no detectable cancer signal in these two mice, so they could be considered "clinically cured."

meet criteria of minimum residual disease (MRD) carried by a substantial proportion of individuals with B cell malignancies in the clinic.[18,19] Therefore, we started the MC.CD20 treatment at this point in time in all later therapy experiments.

Anticancer effects of one i.m. dose of MC.CD20 in a xenograft mouse model

An experiment was conducted to determine the anticancer efficacy of one i.m. dose of MC.CD20 based on the pilot experiments. Fifteen 5-week-old female NOD/SCID mice were injected intraperitoneally (i.p.) with 3 daily doses of cyclophosphamide (CTX) (20 mg/kg), followed by inoculation of Raji.Luc cells, 5×10^5, through tail vein injection. Subsequently, the mice were randomly divided into 3 groups of 5 each. Two days after inoculation of the Raji.Luc cells, one group received one i.m. dose of MC.DNA, 3 µg MC.CD20, and 2.25 µg GL in 50 µL of PBS each, into the *rectus femoris* of both legs. The treat-

ment was not started until the next day, when human T cells were injected intravenously. At this point in time, the Raji cells had already established microcancers in the bone marrow, as illustrated by immunohistology using anti-human CD20 as the primary antibody (Figures 3C–3I). The effector cells were given every other day (q2d), 8 doses in total (Figure 4A). The therapeutic effect was evaluated using bioluminescence intensity, which is approximately proportional to the tumor burden of individual mice, periodically captured by the Caliper Spectrum imaging system (Figure 4B). The Raji cell-generated microcancers expanded exponentially from day 11 after cancer cell inoculation in the groups of Raji cells only or Raji cells plus T cells. Two mice in either of the two control groups showed hindlimb paralysis syndrome, a consequence of nerve damage because of cancer growth, were killed on day 19. The rest of the mice were killed due to the same syndrome on days 25 and 26, respectively. In the MC.CD20 group, however, no obvious bioluminescence signal was

seen until day 17 in one mouse and for an additional two on day 23. Two mice developed hindlimb paralysis syndrome on day 23 and one on day 32. The other 2 mice did not show any signs of cancer development but were sacrificed on day 42 to terminate the experiment.

Tumor burdens were calculated by averaging the bioluminescence intensity of the 5 mice in each group. T cells slowed down Raji cell growth slightly in the T cell-only group, whereas addition of MC.CD20 dramatically retarded the growth (Figures 4B and 4C). The T cell-only group had almost the same survival time curve as the untreated mice, although the tumor burden appeared to be a little lower, suggesting that T cells had little therapeutic effect. In contrast, addition of MC.CD20 significantly reduced the tumor burden and prolonged the survival time (Figure 4D). Importantly, two of the five mice in the MC.CD20 group had no detectable cancer cells throughout the study (Figure 4E), suggesting complete regression or "clinical cure." The evidence suggested that one i.m. dose of MC.CD20 resulted in significant anticancer effects.

Next, the safety of MC.CD20 i.m. injection was evaluated. We detected no systemic release of inflammatory cytokines, such as TNF-α, IFN-γ, and interleukin-12 (IL-12) in the MC.CD20-treated mice (data not shown). In addition, no evidence of muscle and liver toxicity was observed (Figure S4). In a similar experiment, tissues with tumor bioluminescence signal were collected to detect T cell infiltration and tumor cell apoptosis. Strong T cell infiltration was seen in the lungs and spleens of the mice injected with MC.CD20, but not of the two control groups (Figure 5A); frequent apoptotic bodies of tumor cells were also seen in the spleen of the MC.CD20 group, indicating the potent BsAb.CD20-mediated cancer cell killing activity there.

DISCUSSION

Our study demonstrated that one i.m. dose of MC.CD20 could persistently express therapeutic levels of BsAb.CD20, about 200 pg/mL plasma, resulting in significant anticancer effects, with some mice "clinically cured." BsAb.CD20, like BLI, is a simple BsAb with a scFv-linker-scFv format. Interestingly, BLI began to demonstrate anticancer effects at about three times this level, and the efficacy increased as the dosage increased.[20] It will be interesting to examine whether BsAb.CD20 is more effective than BLI (anti-CD3/CD9) in treating B cell malignancies. When treatment was initiated, Raji cell-generated micro-cancers were well established. As seen by immunohistology, these microcancers resemble the MRD in individuals with B cell malignancies seen in the clinic (Figures 3D–3J).[18,19] Therefore, BsAb.CD20 produced by MC.CD20 in the muscle was as effective as i.v. delivered BLI. BLI is the only FDA-approved medicine to treat MRD in individuals with ALL and has been proven to be effective in eliminating the MRD and extending the lifespan.[21] However, the burdens of months-long i.v. administration and high cost limit its clinical acceptance. The results in the present study demonstrated that the i.m. MC.BsAb technology could eliminate these physical and financial burdens without compromising efficacy.

In our earlier proof-of-concept studies, MC.CD20 has been shown to be effective in treating human B cell lymphoma in a similar mouse xenograft model, but the MC vector was delivered through hydrodynamic tail vein injection, which is not applicable to humans.[11] In the present study, MC.CD20 was injected i.m., the most frequent drug administration route in the clinic. Thus, our results suggest that cancers could be treated as conveniently as injecting a vaccine. Recently, the FDA approved two additional BsAbs (both anti-CD20/CD3) for treating B cell malignancies, including the mosunetuzumab with Breakthrough Therapy Designation (BTD) status for treating adults with relapsed or refractory (R/R) follicular lymphoma,[22] and REGN1979 with Orphan Drug Designation (ODD) status for treatment of diffuse large B cell lymphoma (DLBCL) and follicular lymphoma (FL).[23] With a substantial increase in circulation half-life, these two immunoglobulin G (IgG)-like BsAbs can be injected i.v. every 21 days, partially eliminating the physical burden of long-term continuous i.v. injections of BLI. However, they still have the drawback of the inconvenience of frequent hospital visits. More importantly, their molecular size is several times bigger than that of the scFv-linker-scFv BsAbs, so it remains questionable whether they have a comparable tumor penetration capacity and efficacy, especially in eliminating MRD, an important parameter for long-term remission.

The pBB-mediated silencing effect was discovered previously in the mouse liver.[9,24] Our data in the present study confirmed that it occurred in skeletal muscle as well. This confirmation is important because the gene therapy community has long been trying to use muscle as a factory to generate vector-encoded therapeutic gene products, but efforts have been hampered by the short duration of transgene expression using standard pDNA. The MC DNA vector may help accelerate the nonviral vector to enter the clinic.

It has been demonstrated that an adeno-associated virus (AAV) vector genome remains functional in human muscle 10 years after delivery, although almost all of the viral genome remains episomal.[25] Although we demonstrated in this study that luciferase expression from one i.m. dose of MC.Luc lasted up to 343 days in mouse skeletal muscle, it is reasonable to expect that i.m. injected MC.CD20 could also be functional as long as the AAV genome in human muscle and that many diseases can be treated using the i.m. MC technology established in the present study. Importantly, the durability of expression of MC DNA vector-encoding products may significantly enhance BsAb-based anticancer immunotherapy. There is evidence suggesting that sustained remission in individuals with acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), and probably in some individuals with lymphoma, treated with CAR-T cells is associated with persistence of the cellular vectors.[5,26,27] However, it has been shown that the CAR-T cell capacity for expansion, proliferation, and sustainability varies greatly among individuals, so the long-term remission rate is far from satisfactory.[26,28] The i.m. MC.BsAb technology can significantly improve the therapeutic effects because it can be managed to generate an optimal level of the BsAb and maintain it until complete elimination of the MRD. This may
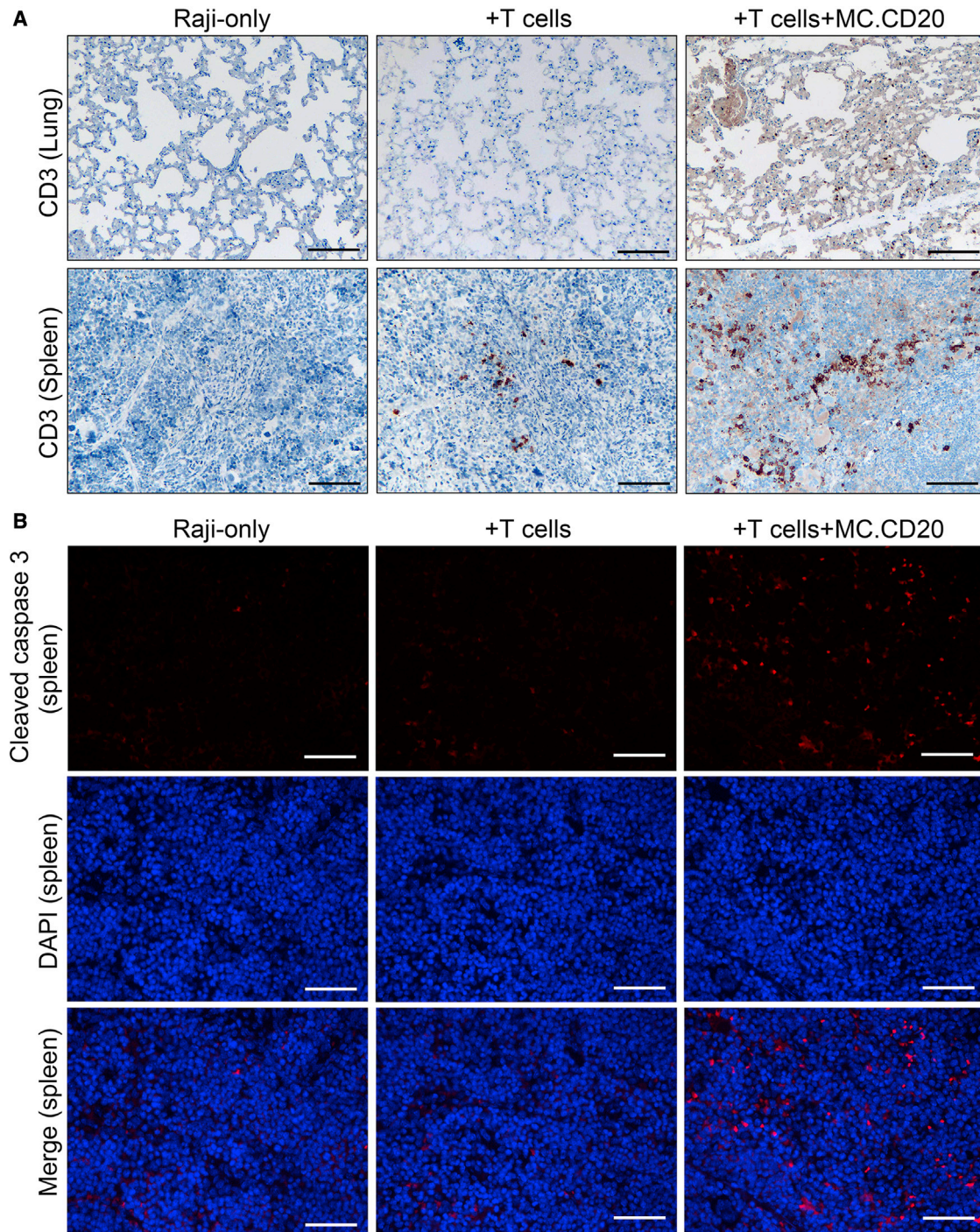
Figure 5. T cell infiltration and tumor cell apoptosis detection

(A) NOD/SCID mice were injected i.v. with Raji.Luc cells after 3 daily i.p. doses of cyclophosphamide. On day 4, the mice received one i.m. dose of MC.CD20 into the *rectus femoris* of both legs. Beginning on day 5, mice received 3 i.v. doses of human T cells q2d. Lung and spleen tissues were harvested for immunohistochemical staining with CD3 antibody after the third injection of T cells. Representative images are shown. Scale bars, 100 μm. (B) Representative immunofluorescence images of human cleaved caspase-3 (red) and nuclei (DAPI, blue) in mouse spleens. Scale bars, 50 μm.

give rise to even better results than those in the present study, in which the optimal level and maintenance time of BsAb might not yet have been reached. This hypothesis is consistent with a report showing that, in a subgroup of individuals with non-Hodgkins lymphoma treated with 4 cycles of continuously i.v. infused BLI, the overall survival rate was more than 50% 8–10 years after the treatment, suggesting a curative effect in a large percentage of individuals.[3]

Although the present study demonstrated that i.m. injected MC.CD20 may stand out as a good choice for treating B cell malignancies, it has the same side effects as CAR-T cells and BLI, such as cytokine release syndrome (CRS) and neurotoxicity. These problems, however, may be less of a concern for i.m. injected MC.CD20 because it can be delivered using the optimized stepwise-dosing strategy established in BLI trials to alleviate the side effects.[19] Medicines such as corticosteroids[19] and anti-CD20 mAbs[22] could prevent them, and several mAbs, such as anti-IL-6 or anti-TNF-α,[22] are effective in treating these toxicities. Target-loss-related escape and resistance are common in all monotarget therapies, and i.m. MC.BsAb technology may overcome this by using the same strategy targeting a second or even third cellular surface antigen simultaneously. Persistent B cell depletion, hypogammaglobulinemia, and related increased infections may be more serious problems, although Ig supplementation is an effective solution. A better solution will be to refine an optimal i.m. MC.BsAb protocol to completely erase cancer cells in a short period of time, like that in the BLI studies,[3] and then withdraw the treatment to allow full recovery of the circulating B cells and Igs.

MATERIALS AND METHODS

Cell lines and mice

Expi293F cells were obtained from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in serum-free FreeStyle 293 expression medium (Thermo Fisher Scientific, Waltham, MA, USA) in a humidified atmosphere of 5% CO_2 at 37°C. The Raji.Luc cells were obtained from Lechen Biotechnology (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 2 mM L-glutamine (Gibco) in a humidified atmosphere of 5% CO_2 at 37°C. Five-to six-week-old female NOD/SCID and BALB/c mice were used for all experiments (Charles River Laboratories, Beijing, China). The mice were maintained in an individual ventilation caging (IVC) system under standardized environmental conditions (20°C ± 1°C room temperature, 50% ± 10% relative humidity, 12-h light-dark cycle) and received autoclaved food and bedding and acidified (pH 4.0) drinking water *ad libitum*. All mouse studies were conducted in accordance with the national guidelines for the humane treatment of animals and were approved by the Animal Care and Experimentation Committee of the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

Antibodies and reagents

Monoclonal mouse anti-human CD20cy clone L26 (IR604) was from Dako (Agilent Technologies, Santa Clara, CA, USA). Anti-CD3 epsilon antibody (CAL54), ab237707), anti-cleaved caspase-3 antibody (E83-77, ab32042), and goat polyclonal secondary antibody to rabbit IgG-heavy & light chain (H&L) (Alexa Fluor 647, ab150079) were purchased from Abcam (Waltham, MA, USA). The CellTrace Violet Cell Proliferation Kit (C34571) and CellTrace Far Red Cell Proliferation Kit (C34572) were purchased from Thermo Fisher Scientific. GL (50531-10G), a natural small molecule, was from Sigma-Aldrich (St. Louis, MO, USA). A mixture of MC.DNA and GL (weight ratio, 4:3) was used as an i.m. injection.

Construction, expression, and purification of BsAb.CD20

The anti-CD3/CD20 expression cassette was inserted into the empty plasmid pMC.BESXP, generating the MC-producing parental plasmid pMC.anti-CD3/CD20. *Escherichia coli* strain ZYCY10P3S2T, transformed with a parental plasmid, was incubated in 200 mL Terrific Broth (TB) medium at 37°C with shaking at 250 rpm for 16 h. After adding induction medium (200 mL of Luria Bertani (LB) medium, 200 μL of 20% L-arabinose, and 8.0 mL of 1 N NaOH) and incubation at 32°C with shaking at 250 rpm for 5 h, MC.CD20 was isolated using a commercially available affinity column (QIAGEN, Hilden, Germany). Purified BsAb.CD20 was a contract product made by Synbio Technologies (Suzhou, China). Briefly, Expi293F cells were transiently transfected with MC.CD20 and cultured in Expi239 expression medium (Gibco). BsAb.CD20 was purified with His Tag purification resin (Roche).

In vitro stimulation and expansion of T cells

Whole blood was donated by a healthy 32-year-old male Chinese individual with written consent. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by Ficoll separation. Dynabeads Human T-Activator CD3/CD28 (Life Technologies, Norway) was added to the PBMCs as recommended by the manufacturer. 5×10^6 isolated PBMCs in a 25-cm^2 cell culture flask were stimulated with Dynabeads Human T-Activator CD3/CD28 at a bead-to-cell ratio of 1:1 in lymphocyte serum-free medium KBM 551 (Corning, NY, USA) preserved 5% FBS and 300 IU/mL of recombinant human IL-2 (R&D Systems, Minneapolis, MN). The stimulation procedure was performed at 37°C and 5% CO_2 for different periods of time.

Determination of immune synapse formation

CellTrace Violet and CellTrace Far Red dye were made separately at stock concentration of 5 mM (20 μL, anhydrous DMSO) and 1 mM (20 μL, anhydrous DMSO). Raji and T cells, 1×10^6 each, were incubated with 1 μL CellTrace Violet and 1 μL CellTrace Far Red, respectively, at 37°C for 20 min with a light shield. Occasionally swirling the cells to prevent settling helped to produce more uniform labeling. We added five times the original staining volume of complete culture medium to the cells and incubated for 5 min. Then we pelleted the cells by centrifugation and resuspended them in fresh pre-warmed complete culture medium. BsAb-mediated immune synapse formation reactions (200 μL) were initiated by combining the two kinds of cells at a 1:1 ratio with or without BsAb.CD20 (40 ng/mL) at 4°C. Aliquots were removed from the two reactions after incubation for 0, 5, 10, 30, 60, and 120 min to determine the frequency of

immune synapse formation by flow cytometry (CytoFLEX, Beckman Coulter Genomics).

BsAb.CD20-redirected T cell cytotoxicity

BsAb.CD20 at various concentrations was incubated with target cells and T cells at an effector-to-target ratio of 4:1 for 4 h. Cytotoxicity was measured by lactate dehydrogenase (LDH) release assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI, USA). After 4 h of incubation, the medium supernatants were collected and measured immediately for LDH activity following the manufacturer's instructions. The absorbance values were measured at 492 nm. The percentage of cytotoxicity was calculated according to the following formula: cytotoxicity percentage = $100 \times$ ([experimental-effector spontaneous-target spontaneous]/ [target maximum-target spontaneous]).

Measurement of cytokine release by ELISA

T cells were mixed with target cells at an effector-to-target ratio of 4:1 and incubated with 10-fold serial dilutions of BsAb.CD20 in U-bottom 96-well plates with 200 μL of RPMI 1640 medium containing 5% FBS; this was done in triplicate. After 4 h, the top 150 μL of medium was transferred to a V-bottom 96-well plate and centrifuged at $200 \times g$ for 5 min to pellet any residual cells. The top supernatant was transferred to a new 96-well plate and analyzed by ELISA for human IFN-γ (ab236895) and TNF-α (ab181421) levels using a kit from Abcam according to the manufacturer' protocol.

Optimization of the MC.DNA i.m. injection protocol

Female 5-week-old BALB/c mice were used to establish the *in vivo* MC.DNA delivery protocol. The optimal dosage of MC.Luc was determined first. A series of mixtures containing different amount of MC.Luc from 0–10 μg and GL at a ratio of 4:3 was made, and a 100-μL aliquot from each mixture was injected into the right *rectus femoris* of groups of mice. 2 days after DNA delivery, individual mice were injected i.p. with a dose of luciferin (3 mg/200 μL) 5–10 min before determination of bioluminescence intensity using the Caliper Spectrum imaging system. Subsequently, groups of mice received an i.m. dose of 3 μg MC.Luc and 2.25 μg GL in 30, 50, 100, and 150 μL of PBS into the left *rectus femoris*, and the biofluorescence intensity was determined 3 days later.

Pharmacokinetics profile

Female NOD/SCID mice were used to determine the serum level of BsAb produced from i.m. injected MC.CD20. The mice received one i.m. dose of MC.CD20, 3 μg mixed with 2.25 μg of GL in 50 μL PBS each, into the *rectus femoris* of both legs. Retro-orbital blood was collected 1, 3, 7, 15, and 30 days after DNA delivery. The serum concentration of BsAb was measured by a TDCC assay. Briefly, 30,000 Raji.Luc cells and 120,000 T cells were added sequentially to each well of the 96-well plate, followed by addition of different amounts of purified BsAb.CD20 (Synbio Technologies) or diluted serum with a final volume of 200 μL/well. The reactions were incu-

bated for 4 h at 37°C, 5% CO_2 before bioluminescence measurements. The luciferase activity, emitted from the surviving Raji.Luc cells, of individual wells was monitored using the Caliper Spectrum imaging system. A standard curve of BsAb.CD20-mediated T cell killing of Raji cells was composed for the TDCC assay to determine the serum BsAb.CD20 level in mice.

Determination of the anticancer efficacy of i.m. injected MC.CD20 *in vivo*

Female NOD/SCID mice, 5–6 weeks old, were conditioned with 3 daily i.p. doses of cyclophosphamide (20 mg/kg each, CTX, Sigma-Aldrich) from days −3 to −1, followed by one i.v. dose of 5×10^5 Raji.Luc cells on day 0. Subsequently, the mice were divided randomly into 3 groups of 5 mice each. On day 2, one group of mice received one i.m. dose of MC.DNA, 3 μg MC.CD20, and 2.25 μg GL in 50 μL of PBS each into the *rectus femoris* of both legs. Beginning on day 3, 5 untreated mice and the MC.CD20-treated mice received 8 i.v. doses of human T lymphocytes q2d, $1 \times 10^7/200$ μL PBS each. Tumor growth was monitored by periodic bioluminescence imaging. Mouse health status was evaluated daily from day 10 after DNA infusion. Mice that developed hindlimb paralysis syndrome were killed by cervical dislocation. The mouse tissue samples were sent to Servicebio Technology (Wuhan, China) for histology services.

Histology

Hematoxylin and eosin (H&E), immunohistochemistry, and immunofluorescence analyses were performed on NOD/SCID mouse tissue. The freshly collected liver, lung, spleen, muscle, vertebra, and femur specimens were fixed in 4% paraformaldehyde solution and then embedded in paraffin. After fixation, the vertebrae and femora were decalcified by ethylenediaminetetraacetate solution for at least 2 weeks. Four-micrometer sections were cut from each paraffin-embedded tissue. Paraffin sections of liver and muscle tissue were stained with H&E solution and then observed by light microscopy for histological changes. For immunofluorescence staining, the sections were incubated overnight at 4°C with anti-cleaved caspase-3 antibody. Following incubation with the primary antibody, sections were incubated for 1 h at room temperature (RT) with Alexa Fluor 647 goat polyclonal secondary antibody to rabbit IgG. Cell nuclei were stained with DAPI solution (1:1,000) for 5 min. For immunohistochemistry staining, mouse anti-human CD20 antibody and rabbit monoclonal anti-CD3 were used as the primary antibodies to illustrate the CD20-positive Raji.Luc cells and CD3-positive T cells with the horseradish peroxidase protocol (DAB; Sigma-Aldrich), respectively. The stained sections were scanned with an Eclipse Ti-SR (Nikon, Japan).

Statistical analysis

GraphPad Prism 8 software (La Jolla, CA, USA) was used for statistical analysis. All data are summarized as mean ± SD, combined with a two-tailed Student's t test to compare two groups or one-way analysis of variance (ANOVA) for analysis of more than two groups, followed by a Bonferroni multiple comparisons test. Survival

time was analyzed by the Kaplan-Meier method and log rank test. The differences were considered significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2022.02.014.

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AUTHOR CONTRIBUTIONS

Z.-Y.C. conceived the concept of application of a BsAb expressed from an i.m. injected MC DNA vector for cancer immunotherapy, supervised the project, and edited the manuscript. X.P. designed and conducted the experiments and drafted the manuscript. X.P., P.Z., P.H., and J.L. conducted the experiments. X.H. prepared the minicircle. G.C., P.C., J.Z., C.-Y.H., and Y.-W.X. took part in the critical review of the manuscript.

DECLARATION OF INTERESTS

Z.-Y.C. and C.-Y.H. were the founders of Syno Minicircle Biotechnology Company, and all other authors, except J.Z., who was an employee of the 7th Affiliated Hospital of Sun-Yet Sun University in Shenzhen, were employees of the company.

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