

PiggyBac-mediated Cancer Immunotherapy Using EBV-specific Cytotoxic T-cells Expressing HER2-specific Chimeric Antigen Receptor

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Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes (CTLs) can be modified to function as heterologous tumor directed effector cells that survive longer *in vivo* than tumor directed T cells without virus specificity, due to chronic stimulation by viral antigens expressed during persistent infection in seropositive individuals. We evaluated the nonviral *piggyBac* (PB) transposon system as a platform for modifying EBV-CTLs to express a functional human epidermal growth factor receptor 2-specific chimeric antigen receptor (HER2-CAR) thereby directing virus-specific, gene modified CTLs towards HER2-positive cancer cells. Peripheral blood mononuclear cells (PBMCs) were nucleofected with transposons encoding a HER2-CAR and a truncated CD19 molecule for selection followed by specific activation and expansion of EBV-CTLs. HER2-CAR was expressed in ~40% of T cells after CD19 selection with retention of immunophenotype, polyclonality, and function. HER2-CAR-modified EBV-CTLs (HER2-CTLs) killed HER2-positive brain tumor cell lines *in vitro*, exhibited transient and reversible increases in HER2-CAR expression following antigen-specific stimulation, and stably expressed HER2-CAR beyond 120 days. Adoptive transfer of PB-modified HER2-CTLs resulted in tumor regression in a murine xenograft model. Our results demonstrate that PB can be used to redirect virus-specific CTLs to tumor targets, which should prolong tumor-specific T cell survival *in vivo* producing more efficacious immunotherapy.

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INTRODUCTION

Immunotherapy with tumor-antigen specific cytotoxic T lymphocytes (CTLs) is increasingly becoming an option for the treatment for intractable cancers that resist standard chemotherapies and radiation. T cells use highly specific mechanisms for tumor-cell

recognition that depend on the expression by the tumor of antigens not expressed on healthy tissues. Many tumors inappropriately express fetal antigens such as survivin and carcinoembryonic antigen or testis antigens like MAGE or SSX that may serve as tumor antigens.^{1,2} However, in practice these antigens are difficult to target, first because high affinity T cells are tolerized, naïve or anergized and therefore difficult to reactivate and expand *in vitro* and second because tumors inhibit the processing and presentation of antigens by MHC proteins on the cell surface. Further, not all tumors express known unique antigens.

T cells of any specificity can be retargeted to known tumor antigens by transgenic expression of recombinant antigen receptors. One class of retargeting receptor that recognizes whole antigens on the cell surface are known as chimeric antigen receptors (CARs), because they combine the antigen-binding domains of antibodies with the ζ chain of the T cell receptor (TCR) to induce tumor cell killing.³ Since tumor cells rarely express costimulatory molecules and commonly inactivate professional antigen presenting cells that might otherwise present tumor antigens in an immunostimulatory manner, second generation CARs also contain intracellular signaling domains from costimulatory molecules, such as CD28, OX40, or 41BB to induce antigen-dependent proliferation and cytokine secretion.^{4,5} The cytolytic function of nonspecifically activated T cells (ATCs) can thus be targeted to poorly immunogenic tumors and this strategy is currently being evaluated in clinical trials for lymphoma using CD20 and CD19-CARs, neuroblastoma using CD171 and GD2-CARs and lung and brain tumors using human epidermal growth factor receptor 2-chimeric antigen receptor (HER2-CAR).⁶⁻¹⁰ So far retroviral and lentiviral vectors have been used for gene transfer in clinical trials.

HER2 is overexpressed on a range of tumors including ovarian cancer, gastric cancer, lung cancer, and breast cancer, and has been a successful target of trastuzumab (Herceptin) antibody therapy.¹¹⁻¹⁴ However, many tumors express HER2 at levels ineffectively recognized by Herceptin.¹⁵ We have previously reported that CD3-ATCs redirected to HER2 by expression of a HER2-CAR

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from a retroviral vector could recognize even low levels of antigen on HER2-positive glioblastoma, osteosarcoma or medulloblastoma cells, efficiently kill the tumor cells *in vitro*, and reduce the tumor burden in xenogeneic murine models.^{16,17}

Lack of T cell expansion and persistence is a major hurdle of T-cell therapy. Even when expressing costimulatory domains from CARs, T cells may be actively suppressed by tumors that commonly express inhibitory ligands, like TGF β and PD-L1 and recruit inhibitory cell types.¹⁸ Expression of multiple costimulatory domains in CARs may provide additional benefits to T cells, but such modified T cells may become hypersensitive to stimulation with potentially toxic results.^{4,9} Therefore we have evaluated Epstein-Barr virus-specific cytotoxic T lymphocytes (EBV-CTLs) as hosts for CARs, since EBV-CTLs have proven safe in multiple clinical trials and EBV antigens expressed by naturally infected B cells at distant sites may produce a beneficial vaccine effect.^{8,19} Indeed EBV-CTLs expressing GD2-CARs expanded more and persisted longer than similarly transduced, CD3-ATCs in patients with relapsed neuroblastoma.⁸

Transposons are capable of integrating into the human genome and stably expressing transgenes,^{20–24} and are less expensive and easier to manufacture than viral vectors. However, effective transposon modification of antigen-specific T cells has not yet been reported and the *piggyBac* (PB) transposon has not yet been evaluated for immunotherapy in an *in vivo* model. We therefore evaluated the potential of EBV-CTLs expressing a HER2-CAR from the PB transposon to eliminate tumors in a mouse model. The PB transposon system has high gene-transfer efficiency and large coding capacity in mouse primary cells, human cell lines, and inducible pluripotent stem cells.^{21,23} We have recently demonstrated the high efficiency of PB gene transfer into resting human T cells,^{24,25} and shown that PB did not preferentially integrate into or near to proto-oncogenes using genome-wide mapping of PB integration sites, compared to retrovirus and lentivirus.^{22,24} We now show that EBV-CTLs can be modified to express a HER2-CAR using PB and demonstrate that transgenic CTLs can be selected using truncated-CD19 expressed as a second transgene. Finally, we show that HER2-CAR-modified EBV-CTLs (HER2-CTLs) can eliminate HER2-expressing tumor cells both *in vitro* and in a NOD-SCID xenograft model.

RESULTS

Production, selection and expansion of EBV-CTLs expressing HER2-CAR

10×10^6 peripheral blood mononuclear cells (PBMCs) were cultured overnight in IL-7-containing T cell medium (TCM) and then nucleofected with two transposon vectors (pIRII-HER2.CD28. ζ and pIRII- Δ CD19) and with pCMV-PB. One day after nucleofection, PBMC numbers had declined to 3.9 ± 0.6 (range 3.9–4.0) $\times 10^6$ mostly due to the cell damage induced by DNA nucleofection. Surviving cells were stimulated with the autologous EBV-transformed B lymphoblastoid cell lines (LCLs) at a 40:1 ratio of PBMCs to LCL in the presence of IL-4 and IL-7 for 9 days and then restimulated with LCLs at a 4:1 ratio in the presence of IL-15. This combination of cytokines improved the viability and expansion of the transfected T cells (not shown). Since the IL-7 receptor is progressively downregulated on ATCs,

IL-15 was used as a growth and survival factor during the second and all subsequent stimulations. Cell numbers increased up to 49.6 ± 23.7 (range 22.4–65.3) $\times 10^6$ after the second EBV-LCL stimulation (Figure 1b). Phenotypic analysis on day 9 or 10 showed that $24.2\% \pm 4.6\%$ (range 20.7–22.5) of CTLs expressed both HER2-CAR and CD19. On day 16 or 17, the expanded CD19 transgene-expressing EBV-CTLs were selected using anti-CD19 microbeads, and immediately cocultured with EBV-LCLs for further 2 weeks. Prior to selection, $30.9\% \pm 7.8\%$ (range 23.7–39.2) of cells expressed HER2-CAR, and $13.4\% \pm 2.6\%$ (range 11.5–16.4) expressed both HER2-CAR and CD19. After CD19 selection this increased to $47.9 \pm 15.5\%$ (range 36.5–65.5) for HER2-CAR and $36.4 \pm 12.6\%$ (22.8–47.8) for both HER2-CAR and CD19. By day 30 of culture, the selected HER2-CTLs reached 33.3 ± 3.5 (range

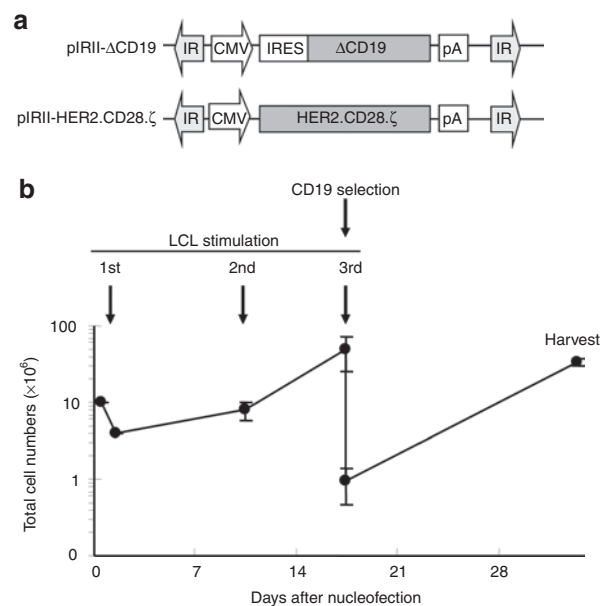


Figure 1 Generation of human epidermal growth factor receptor 2-chimeric antigen receptor (HER2-CAR) expressing Epstein-Barr virus-specific T cells (EBV-CTLs). **(a)** Schema of *piggyBac*-transposons used to nucleofect peripheral blood mononuclear cells (PBMCs). pIRII-HER2.28. ζ and pIRII- Δ CD19 are *piggyBac*-transposon plasmids. pCMV-PB was the *piggyBac*-transposase plasmid. IR, inverted terminal repeat; CMV, cytomegalovirus immediate early promoter; IRES, internal ribosomal entry site; pA, polyadenylation sequence; HER2.28. ζ , Her2/neu-specific chimeric antigen receptor, CD28 and T cell receptor ζ chain; Δ CD19, truncated CD19. **(b)** Production of HER2-CTLs. On day-1, 10×10^6 PBMCs were incubated in interleukin (IL)-7 containing T-cell medium [IL-7-T-cell medium (TCM)] for 20–24 hours. On day 0, IL-7-treated PBMCs were nucleofected with pIRII-HER2.28. ζ pIRII- Δ CD19 and pCMV-PB using the Nucleofector device and immediately transferred into medium containing IL-7-TCM for 20–24 hours. On day 1, to generate EBV-CTLs, nucleofected PBMCs were stimulated with 40 Gy γ -irradiated autologous EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) at a responder: stimulator ratio of 40: 1 in IL-4/IL-7-TCM. On day 9 or 10, the cells were restimulated with LCLs at a 4:1 ratio in IL-15-TCM. On day 16–17, EBV-CTLs were selected for CD19 expression using anti-CD19 MACS beads, immediately transferred in 30 ml of IL-15-TCM in a gas-permeable cell culture device (GRex) and stimulated with 5×10^6 autologous LCLs. On day 30–31, EBV-CTLs were harvested, analyzed, and cryopreserved. The total cell numbers of HER2-CAR- and Δ CD19-nucleofected EBV-CTLs were determined using trypan blue exclusion on day 1, day 9–10, day 16–17, and day 30–31. Data shows the mean \pm s.d. of experiments from three donors.

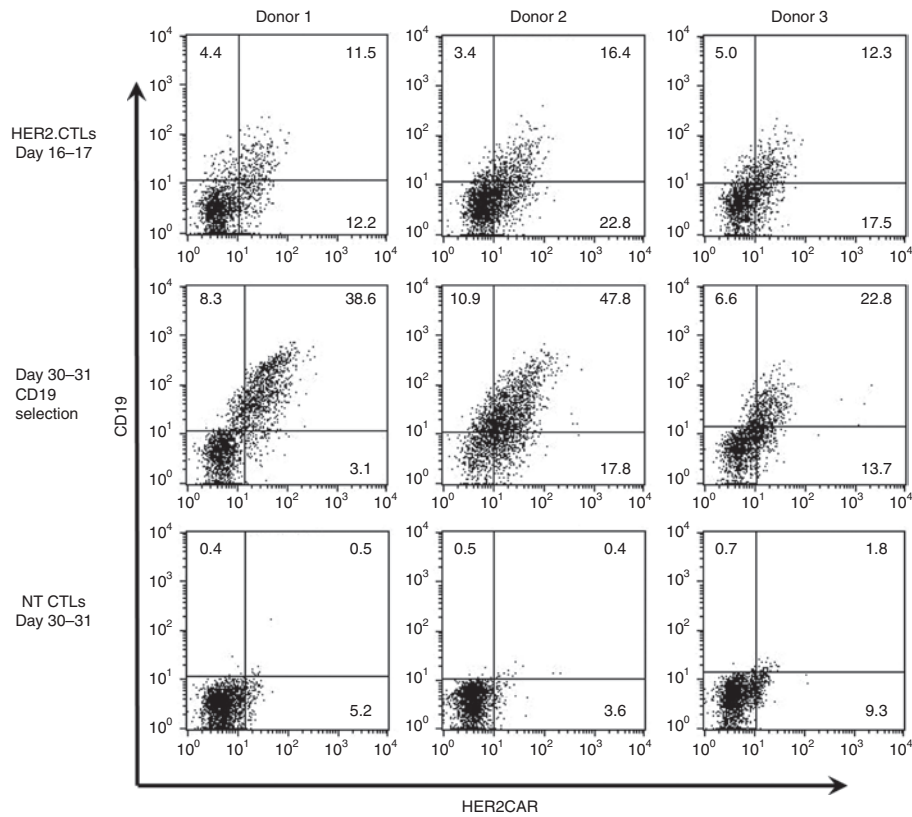


Figure 2 Human epidermal growth factor receptor 2-chimeric antigen receptor (HER2-CAR) expression on nucleofected EBV-CTLs. HER2-CAR- and Δ CD19-nucleofected EBV-CTLs (HER2-CTLs) were analyzed for transgene expression on day 16–17 (before selection) and on day 30–31 (after selection). Nontransfected EBV-CTLs (NT CTLs) were also analyzed as controls. Shown are results from three separate donors. CTLs, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; NT, nontransfected.

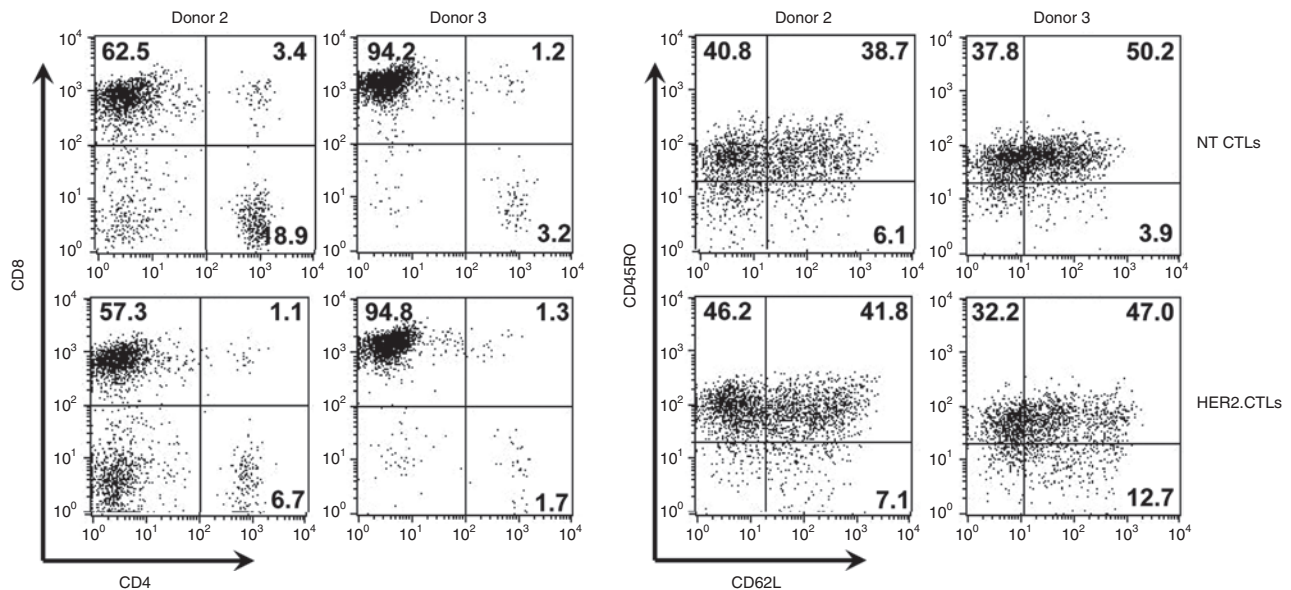


Figure 3 Immunophenotype of HER2-CTLs selected and expanded *in vitro*. Immunophenotyping using anti-CD4, anti-CD8, anti-CD45RO and anti-CD62L antibodies was performed after selection and expansion of HER2-CTLs *in vitro*. Shown are representative results from donor 2 and donor 3 of four donors. CTLs, cytotoxic T lymphocytes. CTLs, cytotoxic T lymphocytes; HER2, human epidermal growth factor receptor 2.

$30.0\text{--}36.9) \times 10^6$ (Figure 2). Thus PB-transposed HER2-CAR-expressing EBV-CTLs can be produced, expanded, and enriched using magnetic-beads.

PB modification did not markedly affect the phenotype of EBV-CTLs: The CD4:CD8 subset ratio in HER2-CTLs did not differ greatly between stably transfected and nontransfected (NT) EBV-

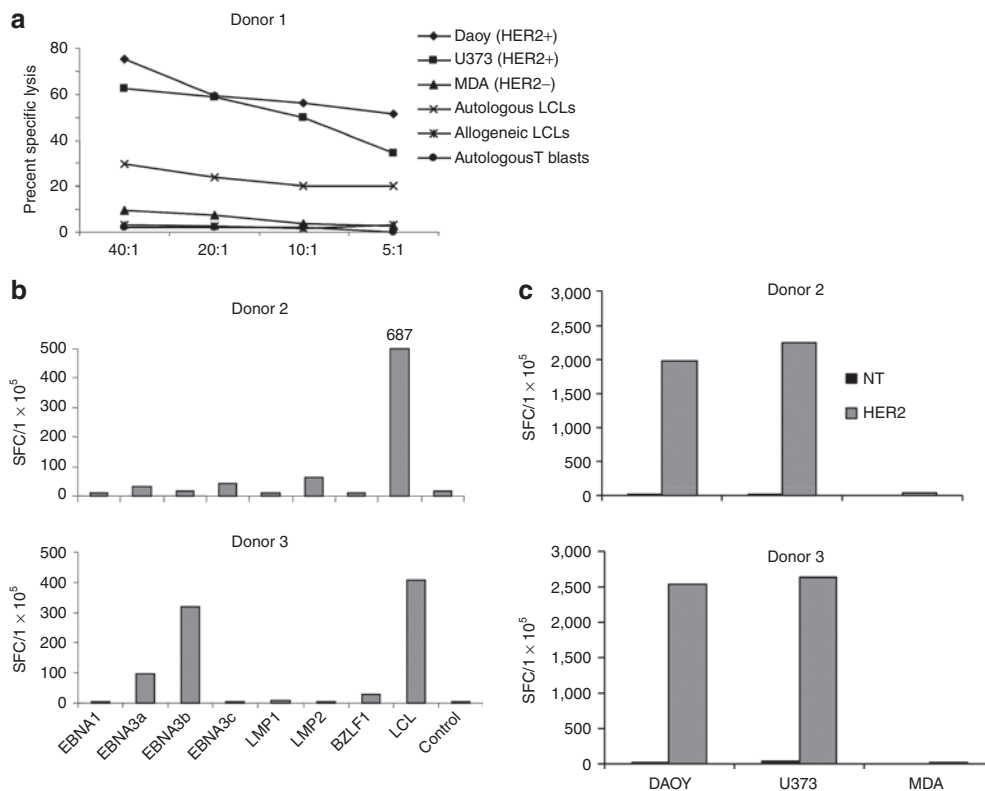


Figure 4 Antigen specificity of HER2-CTLs. After CD19-selection and 14 days-expansion *in vitro*, HER2-CTLs were tested for their ability to target HER2-positive tumor cell lines (Daoy and U373), a HER2-negative tumor cell line (MDA-MB-468), and autologous and allogeneic EBV-LCLs. (a) Cytotoxic specificity for Daoy, U373, MDA, autologous and allogeneic EBV-LCLs, and autologous activated T blasts using Cr⁵¹ release assay. NT EBV-CTLs were tested as controls. (b) Interferon (IFN)- γ production by HER2-CTLs in response to representative EBV antigens, EBNA1, EBNA3A, 3B, 3C, LMP1, LMP2 and BZLF1, and autologous LCLs was measured using ELISPOT assays. (c) IFN- γ production in response to HER2-positive tumor cell lines (Daoy and U373) and HER2-negative tumor cell line (MDA) using ELISPOT assay. NT EBV-CTLs were used as controls in all assays. Similar results were obtained in four donors. CTLs, cytotoxic T lymphocytes; EBV, Epstein-Barr virus; HER2, human epidermal growth factor receptor 2; LCLs, B lymphoblastoid cell lines; NT, nontransfected.

CTLs (Figure 3). About 40% of HER2-CTLs expressed an effector memory phenotype characterized by both CD45RO and CD62L and was similar to the frequency observed in NT EBV-CTLs (Figure 3).

HER2-CTLs specifically target both HER2 antigen and EB virus antigens

To confirm coexisting HLA-independent HER2-specific killing and HLA-restricted EBV-specific killing we performed 4 hour cytotoxicity assays, using HER2 and EBV antigen-expressing tumor cells labeled with ⁵¹Cr. HER2-CTLs showed superior lysis of HER2-positive tumor cell lines, U373, and Daoy (Figure 4a) compared to NT EBV-CTLs (not shown) and equivalent killing of autologous EBV-LCLs. Neither HER2-CTLs nor NT EBV-CTLs killed the HER2-negative breast cancer cell line, MDA-MB-468, allogeneic LCLs, or autologous ATCs (Figure 4a).

The ability of the T cells to produce interferon (IFN)- γ in response to specific antigens was also tested in ELISPOT assays. HER2-CTLs produced IFN- γ in response to autologous EBV-LCLs and to specific EBV antigens presented as peptide mixes by ATCs (Figure 4b) and to HER2-positive tumor cell lines U373 and Daoy (Figure 4c). The NT EBV-CTLs demonstrated a similar pattern of reactivity against the EBV-associated targets but did not recognize the HER2-positive targets. Neither the HER2-CTLs

nor NT EBV-CTLs produced IFN γ when cultured with the HER2-negative tumor cell line, MDA-MB-468. Data from two donors (a total of four experiments) is shown.

We also evaluated the ability of HER2-CTLs to kill tumor targets in coculture assays. HER2-CTLs were cultured with the HER2⁺ glioblastoma cell line, U373, at a ratio of 1: 1 or 2: 1 CTLs to tumor in the absence of cytokines for 5 days. Figure 5 shows HER2-CTLs, NT CTLs, and U373 tumor cells cultured alone and together. HER2-CTLs eliminated virtually all HER2⁺ tumor cells by day 5 both at 2:1 and at 1: 1 ratios, while NT EBV-CTLs did not inhibit tumor growth.

HER2.CAR expression is increased by stimulation through the CAR or the TCR

To evaluate the stability and antigen-inducibility of the transgenic CAR expression from PB, we serially analyzed CAR expression on HER2-CTLs that had received five weekly stimulations with LCLs and were then restimulated (on day 67) through their TCR with EBV.LCLs, through their CAR with HER2-positive tumor cells or with HER2-negative tumor cells and then cultured for 14 days. The expression of the HER2.CAR was then measured over the next 14 days. On day 67 of culture the HER2-CAR was expressed on ~32% of cells with a mean fluorescent intensity

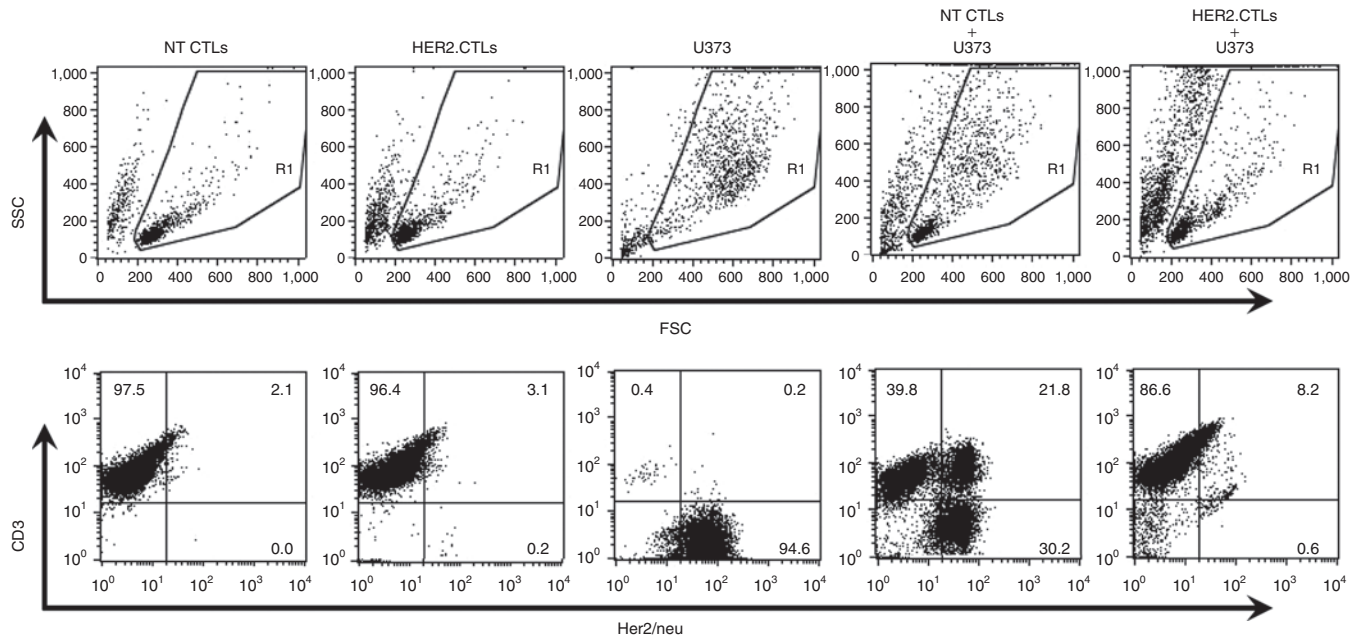


Figure 5 Eradication of HER2-expressing tumor cells by HER2-CTLs. On day 7 after the 4th LCL-stimulation, 1×10^6 HER2-CTLs were cocultured with 1×10^6 HER2⁺ U373 cells. CTLs and U373 cells were plated alone in TCM without cytokines in each well of a 24-well plate as controls. Five days later, cultures were stained with anti-CD3-FITC antibody and anti-Her2/neu-PE antibody and immediately analyzed by flow cytometry. CTLs, cytotoxic T lymphocytes; HER2, human epidermal growth factor receptor 2; LCLs, B lymphoblastoid cell lines.

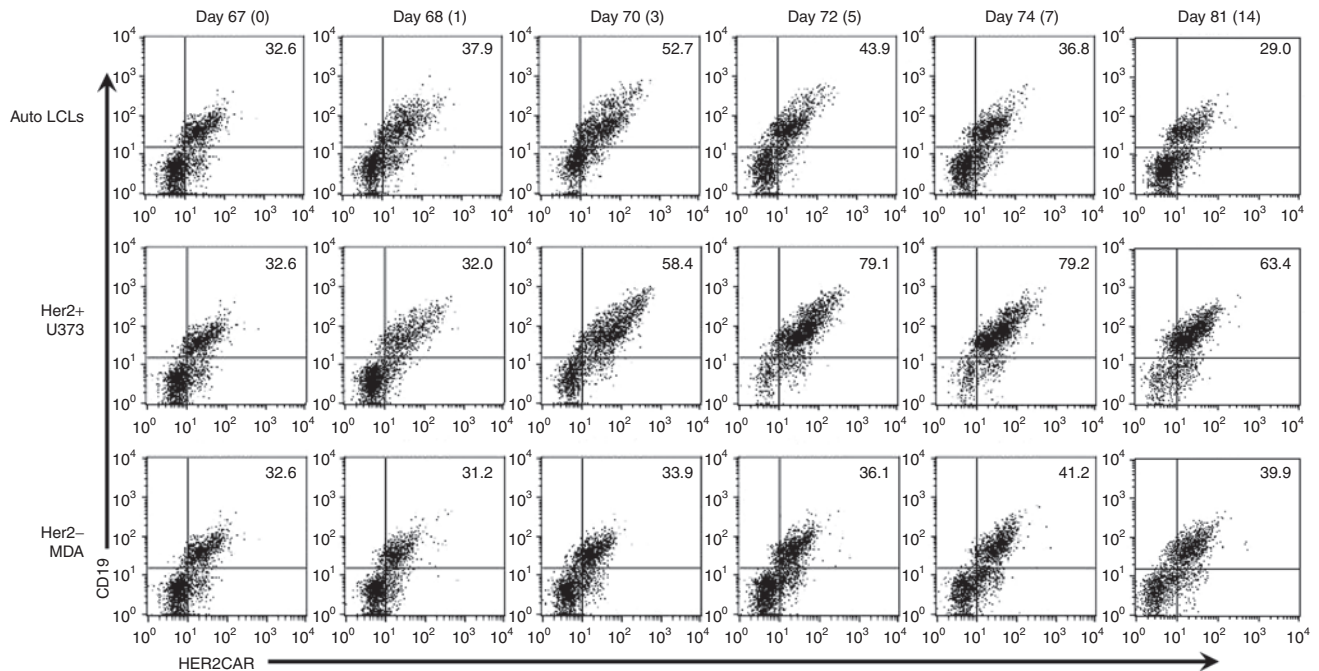


Figure 6 Transient and reversible increase of chimeric antigen receptor (CAR) expression on HER2-CTLs by antigen-specific stimulation. On day 67 of culture, 14 days after the 5th stimulation with autologous LCLs, HER2-CTLs were stimulated with autologous LCLs, HER2-positive U373, or HER2-negative MDA cell lines. HER2-CTLs were analyzed for HER2-CAR- and Δ CD19-expression before the stimulation on day 67 of culture (day 0 prestimulation), and on day 68 (day 1 after stimulation), 70 (3), 72 (5), 74 (7) and 81 of culture (day 14 after stimulation). CTLs, cytotoxic T lymphocytes; HER2, human epidermal growth factor receptor 2; LCLs, B lymphoblastoid cell lines.

(MFI) of 33.3. After restimulation with LCLs, CAR-expression transiently increased (up to 52.7%) but returned to 36.8% by day 8 and 29% by day 14 after stimulation (day 81 of culture) (Figure 6 top panel). By contrast after stimulation through the CAR using

U373 cells as stimulators, transgene expression increased to ~80% by day 5 post stimulation and remained high for up to 14 days (day 81 of culture) (Figure 6, middle panel). There was a slight increase in HER2-CAR expression after stimulation with HER2

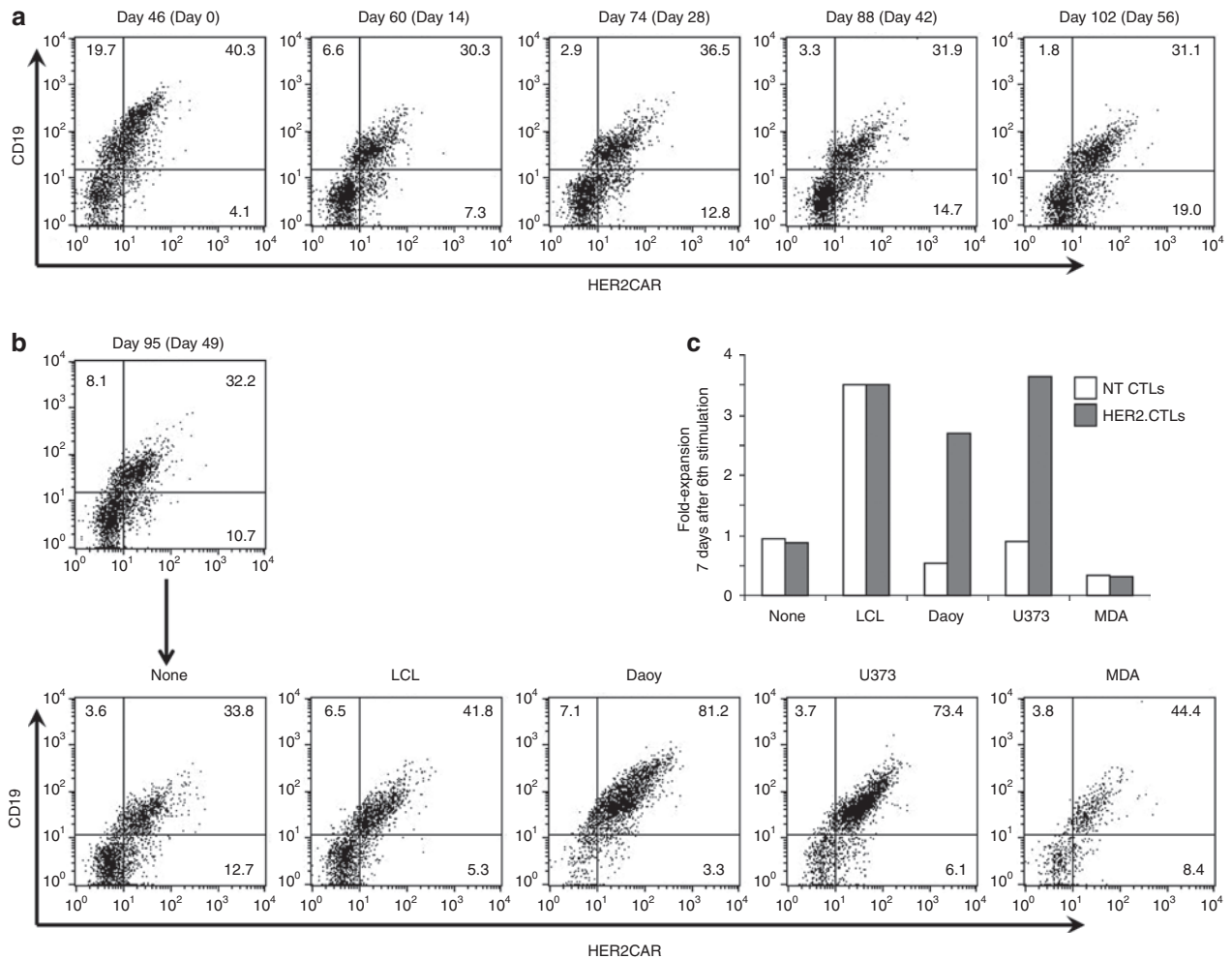


Figure 7 Long term survival and stable chimeric antigen receptor (CAR)-expression of HER2-CTLs *in vitro*. (a) HER2-CTLs received 5th LCL-stimulation at an effector: target ratio of 4: 1 on day 46 post nucleofection, and thereafter they were maintained without any antigen-stimulation in IL-15-TCM. Cells were analyzed for human epidermal growth factor receptor 2-chimeric antigen receptor (HER2-CAR) and Δ CD19 expression every 2 weeks until day 102 post nucleofection. (b,c) On day 95 post nucleofection in a, 1×10^6 cells of 5th-stimulated HER2-CTLs were further stimulated at a 4:1 ratio with autologous LCLs, or HER2 + Daoy or U373 cells or with HER2-MDA cells, or were unstimulated. Seven days later, cells were analyzed for HER2-CAR and Δ CD19 expression by flow cytometry in b and counted using trypan blue exclusion for viable cells in c. As control, NT CTLs were also stimulated with LCLs, analyzed by flow cytometry and counted as same above. CTLs, cytotoxic T lymphocytes; HER2, human epidermal growth factor receptor 2; LCLs, B lymphoblastoid cell lines; NT, nontransfected.

negative MDA cells (Figure 6, lower panel). These results suggest that antigen stimulation via a native TCR or CAR can increase CAR expression from a PB vector, and that significant basal levels were maintained stably in long term culture.

HER2-CTLs survive long-term with stable CAR-expression

To determine how long HER2-CTLs can persist and stably express HER2-CAR *in vitro*, we assessed cell survival and CAR-expression using transfected EBV-CTLs that were maintained in culture without antigenic stimulation but in the presence of IL-15 for 2 months after the 5th stimulation (on day 49) with EBV-LCLs. Surprisingly, HER2-CTLs survived, albeit with minimal expansion for over 100 days after gene-transfer and 56 days after the final LCL-stimulation and continued to express the CAR stably (range 38.0–50.1%) (Figure 7a). Of note, the T cells did not survive in the absence of cytokines (data not shown). To determine if the long-lived HER2-CTLs maintained their antigen specificity and growth ability, we restimulated the

HER2-CTLs with EBV-LCLs or HER2^{+/+} tumor cell lines on day 95 after gene transfer (on day 49 after the 5th stimulation). Seven days later, the long-lived HER2-CTLs showed a dramatic increase of CAR frequency after stimulation with HER2⁺ tumors, U373 and Daoy, (Figure 7b), accompanied by three to fivefold expansion while as expected NT CTLs, which were maintained for equally long in culture, did not respond to HER2-positive tumor stimulation (Figure 7b,c). EBV stimulation induced cell expansion regardless of CAR engineering. These results suggest that HER2-CTLs could survive for several months in the absence of specific stimulation and for longer periods in the presence of EBV-infected cells or HER2⁺ tumor.

Cultured HER2-CTLs are polyclonal

To determine if the transfection and culture procedure resulted in clonal expansion, we analyzed TCR gene rearrangement using the BIOMED-2 multiplex PCR assay in combination with GeneScan analysis, which is a highly sensitive and reliable method in routine testing of T-cell clonality^{26,27} and in which the detection rate of

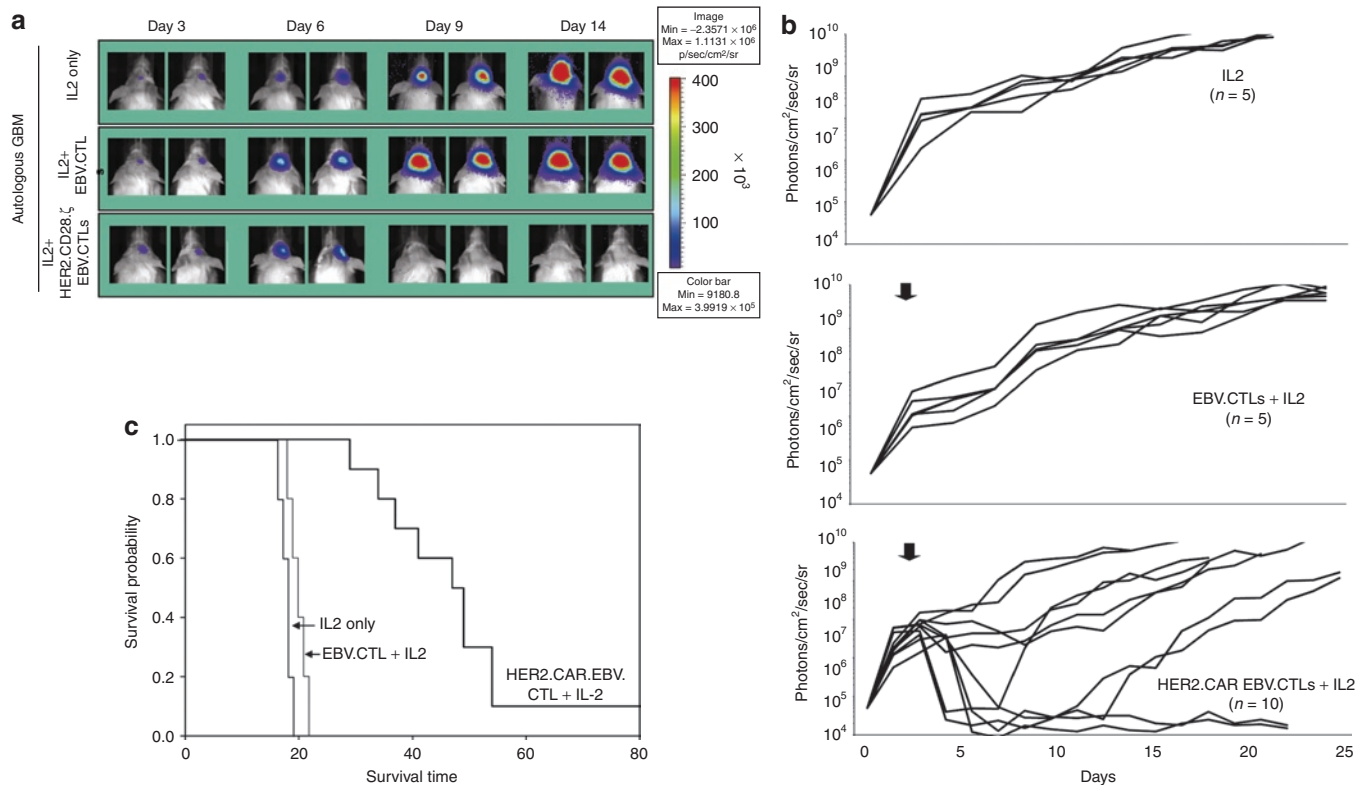


Figure 8 Adoptively transferred HER2-CTLs induce regression of HER2-positive xenografts *in vivo*. 5×10^4 eGFP.FFLuc-expressing HT-1080 cells were injected stereotactically into the caudate nucleus of 9–12 week old SCID mice followed by intratumoral injection of 2×10^6 HER2-CTLs or NT EBV-CTLs on day 6 after tumor inoculation. **(a)** Tumors grew progressively in untreated mice as shown for two representative animals (upper row) and in mice receiving NT EBV-CTLs (middle row), while tumors regressed over a period of 2–5 days in response to a single injection of HER2-CTLs generated from the same donor (lower row). **(b)** Quantitative bioluminescence imaging: HER2-CTLs induced tumor regression when compared to NT EBV-CTLs (two-tailed P value = 0.006, Mann–Whitney U test). Solid arrows: time of T-cell injection; open arrows: background luminescence (mean $\sim 10^5$ photon/sec/cm 2 /sr); n , number of animals tested in each group. **(c)** Kaplan–Meier survival curve: Survival analysis performed 80 days after tumor establishment. Mice treated with HER2-CTLs had a significantly longer survival probability ($P < 0.007$) in comparison to untreated mice and mice that received NT EBV-CTLs. CTLs, cytotoxic T lymphocytes; HER2, human epidermal growth factor receptor 2; EPV, Epstein-Barr virus; NT, nontransfected.

clonal rearrangement of regions of $TCR\beta$ and $TCR\gamma$ ranged from 94 to 98% in patients with T-cell malignancies (see **Supplementary Materials and Methods**)^{28,29} The results of Biomed-2 analysis are shown in **Supplementary Figure S1**. As a control, we used PBMCs from donor 1 and Jurkat cells. GeneScan analyses of the PCR products revealed typical polyclonal Gaussian curves in PBMCs (B) and predominant monoclonal peaks in Jurkat cells (A) for $TCR\beta$ (tube a: 265 nucleotides (nt), tube c: 311 nt) and $TCR\gamma$ (tube d: 212 nt; tube e: 116 nt). EBV-LCLs (C) from donor 1 displayed monoclonal peaks for $TCR\beta$ (tube c: 305 nt) in the absence of T cells ($CD3^+$ cells $< 1\%$). HER2-CTLs (D) from donor 1 (including γ -irradiated EBV-LCLs from donor 1 as stimulator) after a 30-day-culture showed monoclonal peaks for $TCR\beta$ (tube c: 305 nt) in accordance with the regions of TCR gene rearrangement of EBV-LCLs used as antigen-presenting cells for HER2-CTL culture. HER2-CTLs did not show any other monoclonal peaks either for $TCR\beta$ or $TCR\gamma$, but instead showed polyclonal patterns.

Regression of xenografts after administration of HER2.CTL

To evaluate the *in vivo* antitumor activity of HER2-CTLs we used a xenograft model. To allow serial bioluminescence imaging *in*

in vivo, we transduced the human HER2-positive tumor cell line, HT-1080, with a retroviral vector encoding an eGFP-firefly luciferase fusion gene (eGFP.FFLuc). Cells were then sorted for GFP positivity and firefly luciferase functionality was confirmed *in vitro* using a luminometer (data not shown).

5×10^4 eGFP.FFLuc HT-1080 cells were injected stereotactically into the right frontal cortex of SCID mice. On day 6 after tumor-cell injection, mice received an intratumoral injection of 2×10^6 HER2-CTLs ($n = 10$) or NT CTLs from the same donor ($n = 5$). A subset of animals was sham injected ($n = 5$). All animals received 1500U IL-2 intraperitoneally three times weekly for 2 weeks. We quantified tumor growth by serial bioluminescence. In untreated animals, and in animals treated with NT CTLs, tumors grew exponentially over time (**Figure 8a**). In contrast, photon emission decreased significantly in all tested mice after HER2-CTL injection, indicating tumor regression (**Figure 8b**). This was confirmed by histological examination in a subset of animals. Kaplan–Meier survival studies 80 days after tumor injection showed that untreated mice and mice receiving NT CTLs had a median survival of 19 and 20 days respectively. In contrast, mice treated with HER2-CTLs had a median survival of 55 days ($P < 0.007$; **Figure 8c**).

DISCUSSION

We and others have demonstrated that resting human T cells can be transfected stably with transposons.^{24,30} However ATCs are intolerant to transfection and it has been difficult to establish stable transfection of activated antigen-specific T cells. We have now developed a strategy for the successful expression of transgenes in activated, antigen-specific human T cells using the PB transposon system. We used PB to introduce both a HER2-CAR and a selectable marker into PBMCs from which stably transfected, EBV-specific T cells could subsequently be selected and expanded to clinically relevant numbers. We further demonstrated that these CTL could be maintained in culture for over 100 days while retaining stable transgene expression even in the absence of antigenic stimulation. Transgene expression increased transiently in response to stimulation through both the endogenous TCR and the CAR and then returned to baseline. Both EBV antigen-expressing and HER2-expressing tumor cells could be eliminated in *in vitro* assays, while regression of HER2-expressing sarcoma cells could be induced in a NOD-SCID xenograft model.

Manuri *et al.* showed that T cells modified with PB to carry a CD19-CAR could lyse CD19-positive tumor cells *in vitro*.³⁰ These T cells expanded on CD19-expressing artificial antigen presenting cells but the specificities of the native TCRs were unknown. Such CAR-expressing T cells rely on tumor cells and normal B cells to provide stimulatory signals and both of these cell types can be tolerogenic.³¹ To ensure T cell activation upon encounter with tumor a number of groups have evaluated the addition of intracellular signaling domains from costimulatory molecules like CD28, OX40, and 41BB.^{4,5} These molecules may not capitulate the spatio-temporal requirements for T cell activation and may produce T cells that are excessively reactive, with resultant clinical toxicity when multiple costimulatory domains are combined in a single receptor.^{10,32} Further, costimulatory domains may not provide resistance to multiple inhibitory ligands produced by tumors and their accessory cells. An alternative strategy to promote the *in vivo* survival/activity of CAR-expressing T cells could be to use T cells specific for vaccine antigens as carriers for CARs, allowing vaccination after infusion to support T cell maintenance.³³ We have previously investigated the use of EBV-specific T cells as carriers for CARs since these T cells may be stimulated and maintained *in vivo* by "normal" EBV-infected B cells through their endogenous TCRs.^{8,34} Hence when patients with relapsed neuroblastoma were treated with both CD3-activated ATCs and EBV-specific CTLs expressing a GD2-CAR from a retrovirus vector, EBV-specific T cells preferentially expanded and persisted longer than CD3-ATCs after infusion, resulting in three complete remissions among 11 patients treated.⁸

We evaluated the stability of transgene expression after transfection of antigen-specific T cells with PB vectors, but transfection resulted in excessive death of ATCs and we were unable to achieve outgrowth of transgene-expressing CTLs. However when combined with a selectable marker, antigen-specific T cells could be reactivated and then selectively expanded from transfected PBMCs. Methods of transfection other than nucleofection provided no significant advantages (not shown). Surprisingly, if PBMCs were cultured in IL-7 prior to transfection and were then activated with antigen in the presence of both IL-4 and IL-7, the

viability of transgene-expressing antigen-specific T cell precursors could be maintained and we could expand transgenic, antigen-specific T cell lines. Although IL-4 is generally considered a TH2 cytokine, EBV-specific T cells grown in IL-4 and IL-7, maintained their cytolytic activity and their ability to secrete TH1 cytokines (IFN γ and IL-2) following stimulation through both endogenous and transgenic receptors over long term culture. Like many cytokines, the activity of IL-4 is likely to be contextual³⁵ and it appears to have no repolarizing effect on established memory T cells specific for strong viral antigens. The transduced T cells were clearly polyclonal as evidenced by their reactivity with multiple EBV antigens and their polyclonal TCR rearrangements. The single monoclonal TCR V β peak seen in the LCL was unexpected, but TCR gene rearrangement is found in B-cell-derived clonal cells and Biomed-2 protocols have detected TCR gene rearrangement in 35% of precursor B-cell acute lymphoblastic leukemia and 27% of mature B-cell malignancies coincident with immunoglobulin gene rearrangement (although not so far reported in EBV-LCLs).^{36,37}

Retrovirus vectors present several problems for clinical gene therapies. They have a propensity to integrate into or near proto-oncogenes and their prohibitive expense for phase I/II clinical trials, requiring the generation of master producer cell banks and master virus banks. Transposons, by contrast, can be introduced into cells as plasmids at less than 1/10 of the cost. Further, the PB transposon has a large cargo capacity, enabling the introduction of multiple transgenes in a single or multiple transposons,^{21,24,38,39} potentially allowing the expression of additional genes that could protect T cells from tumor-mediated inhibition. The use of any integrating gene delivery system poses the potential risk of genotoxicity when used for human application. We have previously mapped PB integration sites in primary human T cells and found a lack of preference for integrating into or near known proto-oncogenes.^{24,25} Further, we found that the outgrowing T cells had a polyclonal pattern of TCR V β and V γ gene usage, with no indication of mono- or oligoclonality (**Supplementary Figure S1**). Nonetheless, codelivery of a suicide gene can be used for improved safety. We have previously demonstrated PB-mediated delivery of an inducible caspase suicide gene into primary human T cells with chemical dimerizer induced complete cell ablation.^{24,40,41} Our use of a truncated CD19 molecule could also be used for cell ablation via using anti-CD19 antibodies to target transfected cell removal if needed.⁴²

The aim of this study was to develop a nonviral gene-transfer method for EBV-CTLs and we successfully generated EBV-CTLs engineered to express a HER2-CAR using the nonviral PB-transposon system. Up to ~50% of HER2-CTLs expressed an effector memory phenotype which was similar to that of retrovirally transduced EBV-specific CTLs that persisted for up to nine years after infusion into stem cell transplant recipients.¹⁹ Moreover, after infusion into NOD/SCID mice bearing HER2-positive tumors, HER2-CTLs induced tumor regression *in vivo* and resulted in significant extension of the life span of mice harboring tumors. The long term stability of transgene expression is likely to be advantageous for long term tumor control and transient increase in CAR expression after antigen-specific stimulation via native or chimeric TCRs should increase their potency after antigenic challenge.

Using this protocol to generate HER2-CTLs, we obtained $\sim 30 \times 10^6$ dually specific T cells from ~ 10 ml of peripheral blood within about 30 days. In clinical trial for pediatric patients with neuroblastoma using retrovirally GD2-CAR-engineered EBV-CTLs, we infused 2×10^7 to 2×10^8 /m² of CTLs into the patients, which resulted in clinical efficacy and tumor regression. If we collect 30–40 ml peripheral blood from pediatric patients with HER2⁺ medulloblastoma, we could obtain $\sim 10^8$ of HER2-CTLs within 30 days using this protocol. Therefore, engineering of EBV-CTLs to express tumor-specific CARs by PB-transposon system presents an alternative approach to viral vectors for the gene modification of human T cells for therapeutic purposes

MATERIALS AND METHODS

Plasmid construction. The PB-transposase plasmid, pCMV-PB, and PB-transposon plasmid, pIRII- Δ CD19, have been described previously.²⁴ pIRII-IRES- Δ CD19 is a transposon encoding the echomyocarditis virus internal ribosome entry site (IRES) followed by a CD19 gene from which the intracellular signaling domain has been deleted.¹⁶ Both vectors are transcriptionally regulated by the cytomegalovirus (CMV) immediate early gene enhancer/promoter sequence (Figure 1a). We cloned the HER2-CAR with a CD28. ζ signaling domain from a retroviral vector (SFG.HER2.CD28. ζ) (HER2-CAR)⁴³ into pIRII-IRES- Δ CD19 by replacing the IRES- Δ CD19 element with the HER2-CAR (Figure 1a). All plasmid constructs were confirmed by restriction digestion and DNA sequencing.

Blood donors and cell lines. PBMCs from EBV-seropositive healthy volunteers were obtained with informed consent from the Baylor College of Medicine institutional review board. EBV-transformed B lymphoblastoid cell lines (EBV-LCLs) were produced as previously described,⁴³ and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 2 mmol/l L-glutamine (GlutaMAX-I; Invitrogen, Carlsbad, CA). The glioblastoma cell line, U373, the medulloblastoma cell line, Daoy, the sarcoma line HT-1080, and the breast tumor cell line, MDA-MB-468 (MDA) were purchased from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 2 mmol/l L-glutamine for use as target cells in various assays.

Stable transfection of EBV-CTLs with HER2-CAR. We have previously reported efficient gene-transfer of the PB-transposon system into primary human T cells.²⁴ To generate PB gene-modified EBV-CTLs, bulk PBMCs ($>10 \times 10^6$) were cultured overnight in complete T-cell medium (TCM) [Advanced RPMI (Gibco, Gaithersburg, MD)] supplemented with 5% fetal bovine serum and 2 mmol/l L-glutamine containing recombinant human interleukin (IL)-7 (10 ng/ml). The next day 10×10^6 IL-7-treated PBMCs were nucleofected with pIRII-HER2.CD28. ζ transposon (5 μ g), pIRII- Δ CD19 transposon (5 μ g), and pCMV-PB transposase (5 μ g) using nucleofection program U-014 and the Human T-cell Nucleofector Kit (Lonza, Basel, Switzerland). Nucleofected PBMCs were immediately transferred to TCM with IL-4 (1,000 IU/ml) and IL-7 (10 ng/ml) (R&D Systems, Minneapolis, MN), plated into 2 cm² wells of a 24-well plate at 2×10^6 per well for a further 20–24 hours, and then stimulated with 5×10^4 autologous EBV-LCLs that had been irradiated with 40 Gy at a responder: stimulator (R:S) ratio of 40:1 as previously described.⁴³ On day 9 or 10 after nucleofection, EBV-CTLs were counted and restimulated with irradiated autologous LCLs at an R:S ratio of 4:1 in TCM with IL-15 (5 ng/ml) (Proleukin; Chiron, Emeryville, CA). To enrich HER2-CAR-expressing cells, on day 16 or 17, EBV-CTLs were incubated with CD19-microbeads (Miltenyi Biotec, Bisley, UK) for 15 minutes at 4°C, then positively selected using Miltenyi Mini-MACS column according to the manufacturer's instructions. The selected cells were immediately

cocultured with 5×10^6 irradiated autologous LCLs and IL-15 (5 ng/ml) for a further 2 weeks in 30 ml of TCM in a cell culture device with a gas-permeable surface membrane area of 10 cm² (GRex10; Wilson-Wolf Manufacturing, New Brighton, MN).⁴⁴ IL-15 (5 ng/ml) was added to the medium twice weekly. On day 30 or 31, EBV-CTLs were analyzed for HER2-CAR expression and then cryopreserved for further experiments.

Flow cytometry. We analyzed PB transfected EBV-CTLs for transgene expression and phenotype using a FACSCalibur with Cell Quest software (Becton Dickinson, Franklin Lakes, NJ). Cell-surface expression of HER2-CAR was detected with a recombinant human HER2-Fc chimera (R&D Systems). Briefly, stably transfected (HER2-CTLs) and NT EBV-CTLs were incubated with HER2-Fc for 30 minutes at 4°C, and detected with a goat anti-human Fc fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Chemicon, Temecula, CA), peridinin chlorophyll protein (PerCP)-conjugated CD3 monoclonal antibody (MAB) (Becton Dickinson) and allophycocyanin (APC)-conjugated CD19 MAB (Becton Dickinson).

To determine subpopulation and memory phenotype of the generated HER2-CTLs, we stained the cells with phycoerythrin (PE)-conjugated CD4 (CD4-PE) MAB, CD8-APC MAB and CD3-PerCP MAB, CD62L-PE MAB, CD45-RO-APC MAB and CD3-PerCP MAB (all from Becton Dickinson), respectively, and analyzed them by flow cytometry. NT EBV-CTLs and appropriate isotype-matched antibodies were used as controls in each experiment.

Cytotoxicity assay. To determine if HER2-CTLs were able to lyse target cells, we performed cytotoxicity assays using a standard ⁵¹Cr release assay.⁴⁵ As effectors, we used HER2-CTLs or NT EBV-CTLs 7 days after the 4th stimulation, and as targets we used HER2-positive tumor cell lines, U373 and Daoy, a HER2-negative tumor cell line, MDA-MB468, autologous and HLA-mismatched EBV-LCLs and autologous T cells activated with a CD3 antibody (100 ng/ml) (Ortho Biotech, Bridgewater, NJ) (ATCs). ATCs were used as target cells 7–14 days after stimulation in IL-15-containing TCM. Target cells were labeled for 1 hour with ⁵¹Cr sodium chromate (Na₂CrO₄), washed and then incubated with effector cells at an E:T ratio of 40:1, 20:1, 10:1, or 5:1 for 4 hours at 37°C in 5% CO₂ incubator. The percent specific lysis was calculated as (experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100.

Enzyme-linked immunospot assay. We measured interferon (IFN)- γ production by HER2-CTLs in response to stimulation with representative EBV antigens and tumor cell lines using enzyme-linked immunospot (ELISpot) assay. Briefly, transfected and NT EBV-CTLs were serially diluted from 2×10^5 to 5×10^4 cells/well, and we measured the virus-specific activity of responder cells after direct stimulation with autologous EBV-LCL (1×10^5 cells/well) and pepmixes (15 mers overlapping by 11 amino acids from JPT Technologies, Berlin, Germany) spanning the EBV latent and lytic antigens EBNA1, EBNA3a, EBNA3b, EBNA3c, LMP1, LMP2, and BZLF1. All pepmixes were used at a concentration of 100 ng/peptide/well. Tumor-specific activity was measured by incubating responder cells with the HER2-positive tumor cell lines, U373 and Daoy, and a HER2-negative tumor cell line, MDA-MB-468, all plated at 1×10^5 cells/well. Each culture condition was run in duplicate. After 20 hours of incubation, plates were developed as previously described, dried overnight at room temperature in the dark, then sent to Zellet Consulting, New York, NY for quantification.⁴⁵ Spot-forming cells and input cell numbers were plotted, and a linear regression calculated after excluding plateau data points. The frequency of T cells specific to each antigen was expressed as specific spot-forming cells per input cell numbers.

Coculture experiments. To evaluate the ability of HER2-CTLs to eliminate HER2-positive(+) tumors, we cocultured 1×10^6 HER2-CTLs or NT EBV-CTLs (on day 7 after their 4th stimulation with LCLs) with non-irradiated HER2⁺ tumor cell, U373, at effector to target cell ratios of 2:1 or 1:1 in TCM in the absence of cytokines in 24-well plates. As controls, 1×10^6 of

NT or CAR-engineered EBV-CTLs, and 0.5×10^6 U373 were also cocultured. Phenotypic analyses were performed on days 1, 3, and 5. T cells and tumor cells were detected using anti-CD3-FITC-conjugated CD3 MAb and anti-Her2/neu-PE-conjugated MAb by flow cytometry.

NOD-SCID xenograft model. All animal experiments were conducted on a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Recipient NOD-SCID mice were purchased from Taconic (C.B-Igh-1^b/IcrTac-Prkdc^{scid}; FOX CHASE CB-17 SCID ICR; Taconic, Hudson, NY). Male 9- to 12-week-old mice were anesthetized with rapid sequence inhalation isoflurane (Abbot Laboratories, Abbot Park, IL) followed by an intraperitoneal injection of 225–240 mg/kg Avertin solution and then maintained on isoflurane by inhalation throughout the procedure. The head was shaved, mice were immobilized in a Cunningham Mouse/Neonatal Rat Adaptor (Stoelting, Wood Dale, IL) stereotaxic apparatus fitted into an E15600 Lab Standard Stereotaxic Instrument (Stoelting), and then scrubbed with 1% povidone-iodine. A 10 mm skin incision was made along the midline. The tip of a 31G ½ inch needle mounted on a Hamilton syringe (Hamilton, Reno, NV) served as the reference point. A 1 mm burr-hole was drilled into the skull, 1 mm anterior to and 2 mm to the right of the bregma. Firefly-luciferase expressing HER2-positive HT-1080 cells (5×10^4 in 2.5 μ l) were injected 3 mm deep to the bregma, corresponding to the center of the right caudate nucleus over 5 minutes. The needle was left in place for 3 minutes, to avoid tumor cell extrusion, and then withdrawn over 5 minutes. Five days after tumor cell injection, animals were treated with 2×10^6 effector T cells in 5 μ l to the same tumor coordinates. The incision was closed with 2–3 interrupted 7.0 Ethicon sutures (Ethicon, Somerville, NJ). Animals received 1500U IL-2 intraperitoneally three times weekly for 2 weeks. A subcutaneous injection of 0.03–0.1 mg/kg buprenorphine (Buprenex, Hull, England) was given for pain control.

Bioluminescence imaging. Isoflurane anesthetized animals were imaged using the IVIS system (IVIS; Xenogen, Alameda, CA) 10 minutes after 150 mg/kg D-luciferin (Xenogen) was injected intraperitoneally. The photons emitted from luciferase-expressing cells within the animal body and transmitted through the tissue were quantified using “Living Image,” a software program provided by the same manufacturer. A pseudo-color image representing light intensity (blue least intense and red most intense) was generated and superimposed over the grayscale reference image. Animals were imaged every other day for 1 week after injections, then twice weekly for 2 weeks then weekly thereafter. They were regularly examined for any neurological deficits, weight loss, or signs of stress and euthanized according to preset criteria, in accordance with the Baylor College of Medicine’s Center for Comparative Medicine guidelines.

Statistical analysis. The data are presented as mean \pm 1 SD. The Student’s *t*-test was used to determine the statistical significance of differences between samples, and *P* values less than 0.05 were accepted as indicating a significant difference.

SUPPLEMENTARY MATERIAL

Figure S1. Evaluation of T-cell clonality of HER2-CTLs using BIOMED-2 multiplex PCR and GeneScan analysis.

Materials and Methods.

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