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Rejection resistant CD30.CAR-modified Epstein-Barr virus-specific T cells as an off-the-shelf platform for CD30$^+$ lymphoma

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Off-the-shelf (OTS) adoptive T cell therapies have many benefits such as immediate availability, improved access and reduced cost, but face the major challenges of graft-vs-host disease (GVHD) and graft rejection, mediated by alloreactive T cells present in the graft and host, respectively. We have developed a platform for OTS T cell therapies by using Epstein-Bar virus (EBV)-specific T cells (EBVSTs) expressing a chimeric antigen receptor (CAR) targeting CD30. Allogeneic EBVSTs have not caused GVHD in several clinical trials, while the CD30.CAR, that is effective for the treatment of lymphoma, can also target alloreactive T cells that upregulate CD30 on activation. Although EBVSTs express high levels of CD30, they were protected from fratricide *in cis*, by the CD30.CAR. Hence, they could proliferate extensively and maintained function both through their native EBV-specific T cell receptor and the CD30.CAR. The CD30.CAR enabled EBVSTs to persist in co-cultures with naive and primed alloreactive T cells and eliminate activated natural killer cells that can also be alloreactive. In conclusion, we show that CD30.CAR EBVSTs have the potential to be an effective OTS therapy against CD30$^+$ tumors and, if successful, could then be used as a platform to target other tumor antigens.

INTRODUCTION

Autologous T cells have had remarkable success in treating cancers and lack the severe long-term toxicities associated with conventional therapies. In particular, T cells expressing chimeric antigen receptors (CARs) have produced complete responses in patients with B-cell malignancies, while virus-specific T cells (VSTs) have effectively treated virus-associated diseases and malignancies after hematopoietic stem cell transplant (HSCT).[1–5] Major limitations of autologous T cell therapies are the patient-specific manufacturing that is expensive and often fails in patients with immunodeficiency disorders or cancer, and the manufacturing time that excludes patients with urgent need.[6] Banked, off-the-shelf (OTS) T cells from selected healthy donors are rapidly available for any patient, improving accessibility and reducing costs.[7] However, alloreactive T cells that recognize foreign HLA antigens, present the greatest challenge to allogeneic therapies. Alloreactive T cells in the graft may attack host tissues, resulting in graft-vs-host disease (GVHD) and alloreactive T cells in the host reject allogeneic cell therapy products.

The most common approach to prevent GVHD in polyclonally activated T cells (ATCs) expressing CAR, has been to knock out the endogenous T cell receptor (TCR).[8–10] However, patients infused with less than 1% residual TCR-positive T cells can develop GVHD.[11] Alternatively, VSTs, which have a far less diverse TCR repertoire than ATCs, can be used as an OTS platform, since they have rarely produced GVHD in allogeneic recipients[12–14] and have proved safe and effective as a banked allogeneic treatment for viral infections in HSCT recipients.[15,16] The most common strategy to circumvent graft rejection is to knock out human leukocyte antigen (HLA) class I and II molecules.[9,17] However, this relies on gene editing, introducing risks for chromosomal instability, while HLA class I-negative cells are targets for natural killer (NK) cells.[18] We previously explored an alternative approach of eliminating alloreactive T cells by engineering a fusion protein called the chimeric HLA antigen receptor (CHAR) that links beta-2 microglobulin (B2M), the universal component of class I HLA molecules, to the cytotoxic CD3 zeta chain of the TCR. CHAR-modified T cells eliminated engaged alloreactive T cells, but the CHAR induced significant fratricide and required inducible expression.[19] We subsequently showed that a CAR targeting the T cell activation marker, 4-1BB, that is upregulated on alloreactive T cells, protected CD3 and CD28 antibody ATCs from rejection in preclinical models.[20]

Several other activation markers are upregulated on alloreactive T cells after exposure to allogeneic cells and may be targeted with

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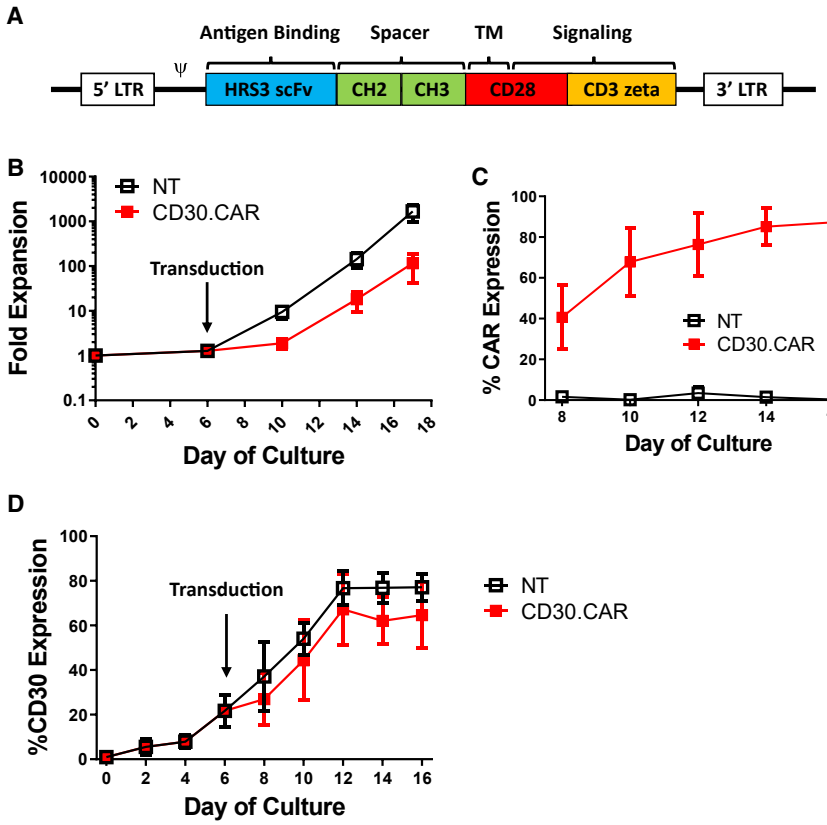

Figure 1. CD30.CAR EBVSTs proliferate selectively in culture despite CD30 expression

(A) Design of CD30.CAR construct in retrovirus backbone. (B) Proliferation of NT EBVSTs and CD30.CAR EBVSTs. Expression of CD30.CAR (C) and CD30 (D) in NT EBVSTs and CD30.CAR EBVSTs. Data denoted as mean ± SEM (n = 3). LTR, long terminal repeats; TM, transmembrane

CARs. CD30, also known as TNFRSF8, is a member of the tumor necrosis factor (TNF) superfamily and is one example.[21–23] Due to its expression on malignancies including Hodgkin lymphoma, anaplastic large cell lymphoma and human T cell leukemia virus type 1^+ T cell lymphoma,[24] CD30 has been targeted with a CAR in clinical studies that demonstrated the safety and efficacy of autologous CD30.CAR ATCs in patients with $CD30^+$ lymphoma.[25,26] Hence, a CD30.CAR could not only protect OTS T cells against allo-rejection but also eliminate $CD30^+$ tumors. Here we show that despite expressing CD30, CD30.CAR-modified Epstein-Barr virus-specific T cells (CD30.CAR-EBVSTs), are resistant to fratricide, proliferate extensively after activation, and exert functions through both the CD30.CAR and their endogenous EBV-specific TCRs. CD30.CAR-EBVSTs resist killing by both resting (peripheral blood) and activated alloreactive T cells. Thus, we present an OTS therapy platform that does not require knock-out gene editing and can readily be translated to the clinic. If effective, the CD30.CAR could be used to prevent rejection of allogeneic T cells expressing heterologous CARs or TCRs targeting other malignancies.

RESULTS

Selective expansion of CD30.CAR-modified EBVSTs

ATCs, including EBVSTs, express CD30; therefore, we first determined if we could generate and expand EBVSTs expressing CD30.CAR. Peripheral blood mononuclear cells (PBMCs) were memory enriched, by depleting the $CD45RA^+$ fraction that contains naive T cells, then stimulated with overlapping peptide libraries (pepmixes) spanning EBV latent and lytic cycle antigens and cultured in medium supplemented with IL-7 and IL-15.[27] The cells were transduced with a second generation CD30.CAR containing a CD28-derived endodomain (Figure 1A) on day 5 or day 6, then re-stimulated on day 9 or day 10 using an irradiated complex of pepmix-pulsed, autologous ATCs (pxATCs) and a $CD30^+$, HLA-negative universal EBV-transformed lymphoblastoid cell line (ULCL), in the presence of cytokines. The cells were cryopreserved for testing between days 16 and 20. Of note, the culture medium was supplemented with human platelet lysate (HPL) since CD30.CAR-EBVSTs grown in fetal bovine serum (FBS) containing medium produced high non-specific interferon gamma (IFN-γ) release in the absence of antigen in the enzyme-linked immunospot (ELISpot) assay (Figure S1A), resulting in significantly reduced antigen-specific responses to EBV after background subtraction (Figure S1B). To mitigate this non-specific background, we screened several different growth conditions (data not shown) and found CD30.CAR EBVSTs grown in medium containing HPL showed considerably less background cytokine production; thus, this HPL medium was subsequently used for the experiments in the study. CD30.CAR-EBVSTs showed an initial delay in expansion compared with non-transduced (NT) EBVSTs, but subsequently recovered and proliferated similarly (Figure 1B). Notably, CD30.CAR expression increased from 40.59% ± 15.76% on day 8, up to 87.25% ± 6.9% at the end of culture (Figure 1C), despite expressing CD30 (Figure 1D), suggesting that CD30.CAR expression instead provided a proliferative advantage or eliminated non-transduced CD30+ T-cells.

CD30.CAR EBVSTs have dual function through the CAR and the TCR

To evaluate CD30.CAR-EBVST effector functions, we performed cytotoxicity assays. CD30.CAR EBVSTs eliminated $CD30^+$ HDLM-2, but not CD30- BJAB tumor cells in a 4–5 h chromium release cytotoxicity assay, while NT EBVSTs showed minimal killing (Figure 2A). CD30.CAR-EBVSTs maintained EBV specificity at similar levels to that of NT EBVSTs as determined by measuring the number of IFN-γ-secreting cells in response to stimulation

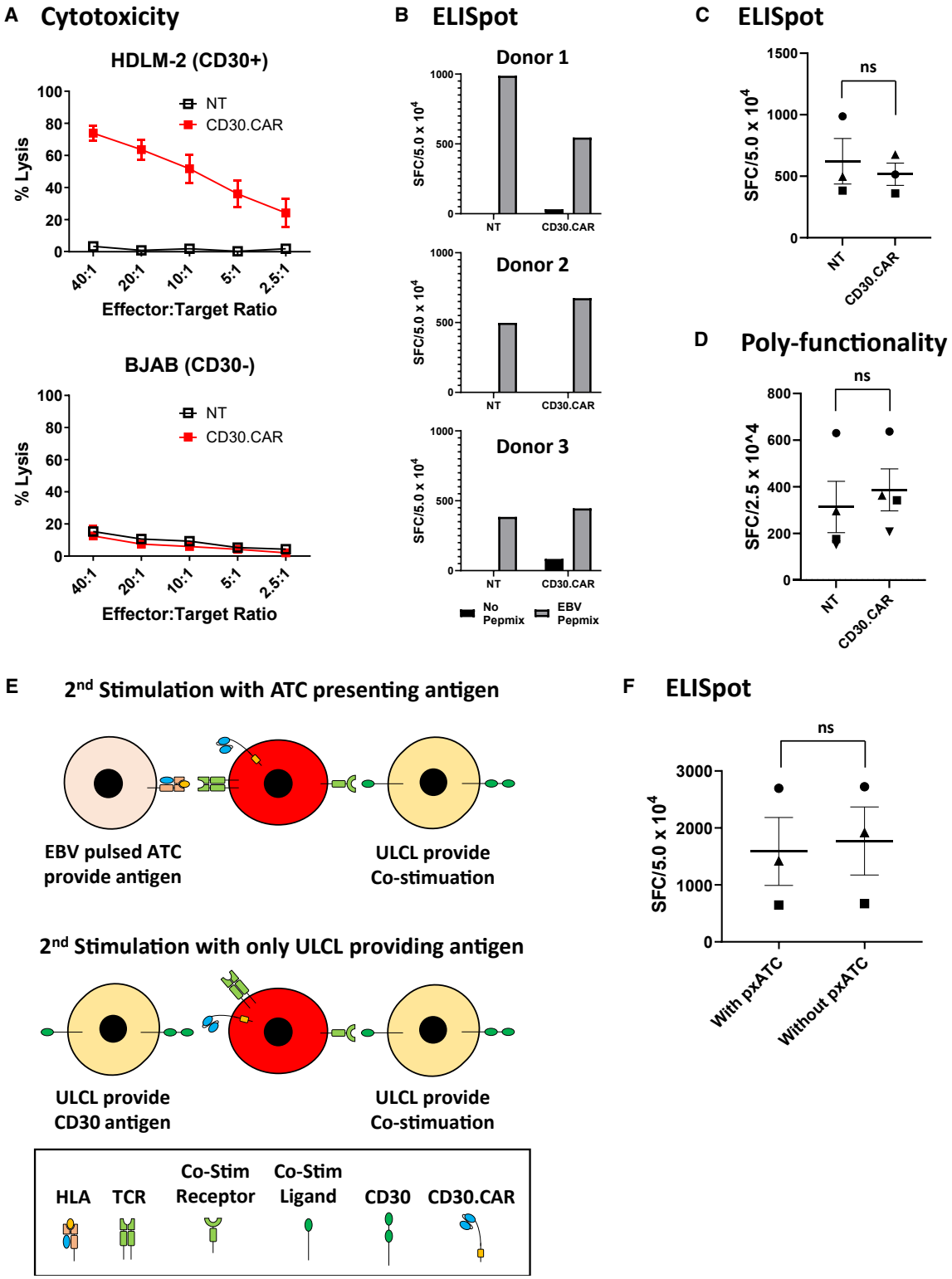

Figure 2. Dual specificity of CD30.CAR EBVSTs

(A) CD30-specific cytotoxicity assessed in a ^{51}Cr chromium release cytotoxicity assay. NT EBVSTs and CD30.CAR-EBVSTs were incubated with either CD30-positive tumor cells (HDLM-2) or CD30-negative tumor cells (BJAB) for 4–5 h and chromium release quantified using a gamma counter (n = 3). (B) EBV specificity was assessed using the

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with EBV pepmixes in the ELISpot assay (Figures 2B and 2C). We next assessed whether the CD30.CAR could affect the poly-functionality of EBVSTs, as determined by the ability of cells to secrete several different effector molecules. Using the FluoroSpot assay that is designed to measure the production of multiple cytokines in a single cell, we found that the frequency of cells secreting IFN-γ, granzyme B, and TNF-α was similar in CD30.CAR EBVSTs and NT EBVSTs (Figure 2D). Maintaining the specificity and expansion of antigen-specific T cells requires antigen-specific stimulation via the TCR from a potent antigen-presenting cell every 7–10 days.[28] To determine if we could replace TCR stimulation of CD30.CAR-EBVSTs by pxATCs with CAR stimulation, without losing antigen specificity, we compared EBV antigen specificity after a second stimulation with the $CD30^+$ ULCL alone or combined with pxATCs (Figure 2E). CD30.CAR-EBVSTs restimulated with or without pxATCs showed similar fold expansion (Figure S2A), CD30.CAR expression (Figure S2B), and cytotoxicity against $CD30^+$ targets (Figure S2C), and there was no significant difference in EBV specificity as determined by the ELISpot assay (Figure 2F).

Expression of the CD30.CAR in *cis* prevents CD30.CAR-mediated fratricide

As CD30 is upregulated on EBVSTs (Figure 1D), it was surprising that CD30.CAR-EBVSTs could expand normally (Figure 1B) and that we did not observe extensive fratricide. Instead, we found that CD30 expression on EBVSTs was highly correlated with expression of the CD30.CAR (Figure 3A), suggesting that CD30-expressing T cells that lacked the CD30.CAR were eliminated. Since downregulation of CD30 was clearly not a mechanism of fratricide resistance (Figure 3A), we hypothesized that, by binding CD30 *in cis*, the CD30.CAR could block its recognition *in trans* by other CD30.CAR-EBVSTs (Figure 3B). To test this possibility, we expressed the CD30.CAR or a truncated, non-cytotoxic derivative lacking intracellular signaling domains, in a $CD30^+$ Hodgkin lymphoma cell line, HDLM-2. HDLM-2 expressing either the CD30.CAR or the truncated CD30.CAR were protected from lysis by CD30.CAR EBVSTs (Figure 3C), indicating that CD30.CAR interacts with CD30 at the cell surface and prevents CD30.CAR directed killing by other CD30.CAR T cells. To evaluate whether *cis* binding increases the baseline activation of CD30.CAR-EBVSTs, we compared the expression of the activation markers CD137, CD134, CD69, CD25, and HLA-DR and exhaustion markers PD-1, LAG-3, Tim-3, TIGIT, and CD39 with that in NT EBVSTs. CD30.CAR-EBVST had significantly higher levels of CD137 and CD25 (Figure S3A) than NT EBVSTs, but no significant differences in exhaustion markers (Figure S3B). To measure baseline cytokine secretion in the absence of stimulation, we used the FluoroSpot assay and found the production of IFN-γ, granzyme B, and TNF-α to be significantly higher in CD30.CAR EBVSTs compared with NT EBVSTs (Figure S3C).

The CD30.CAR protects EBVSTs from alloreactive T cells

To determine if CD30.CAR-EBVSTs could resist alloreactive T cell-mediated killing, we established a mixed lymphocyte reaction (MLR) by combining donor HLA-A2-positive NT EBVSTs or CD30.CAR-EBVSTs with recipient allogeneic HLA-A2-negative PBMCs at a ratio of 1:4 and quantified cell numbers by flow cytometry on days 0, 4, and 12 (Figure 4A). Representative flow plots from day 0 show the presence of donor HLA-A2-positive EBVSTs and recipient HLA-A2-negative allogeneic PBMCs, distinguished by their expression of HLA-A2. Although NT EBVSTs were still present on day 4, they were eliminated by day 12, which corresponded with an expansion of recipient alloreactive T cells (Figure 3B). In contrast, CD30.CAR-EBVSTs survived and had expanded by day 12 and prevented the proliferation of both CD4 and CD8 recipient alloreactive T cells (Figure 3C), but did not eliminate all of the allogeneic cells, showing that CD30.CAR-EBVSTs themselves lacked alloreactivity toward CD30-negative cells.

Kinetics of CD30 expression on ATCs

The delayed allo-rejection kinetics observed in the primary MLR (Figure 4), in which EBVSTs are not eliminated by day 4, likely reflects the time required for small numbers of resting alloreactive T cells to expand and acquire cytolytic function. We, therefore, measured the kinetics of CD30 expression on CD4 and CD8 selected peripheral blood T cells after allo-activation using allogeneic LCLs in the absence of cytokines. As a negative control stimulus, we used the ULCL that lacks surface expression of HLA class I and HLA class II molecules. CD30 could not be detected on either CD4 or CD8 T cells until day 3 after allogeneic LCL stimulation, with peak expression on day 6 before a subsequent decrease on day 8 (Figure 5A). Little to no CD30 was observed in T cells cultured alone or with the ULCL. To account for the difficulty in detecting initial low frequencies of alloreactive T cells in PBMCs by flow cytometry, we poly-clonally activated T cells with plate-bound CD3 and CD28 antibodies (Figure 5B). Strong CD30 expression was detected in both CD4 (59.2% ± 8.8%) and CD8 (63.7% ± 8.94%) T cell, but not until 48 h after stimulation, which is delayed compared with early activation markers, such as CD69, and may explain the delayed elimination of alloreactive T cells.

CD30.CAR EBVSTs resist killing by primed alloreactive T cells and subsequently kill tumor cells

Since CD30 is upregulated with slow kinetics after activation, alloreactive T cells are unlikely to be killed upon their first interaction with CD30.CAR-EBVSTs, but after priming would be fully activated

ELISpot assay. EBVSTs were stimulated with EBV antigens (EBNA1, LMP1, and LMP2) and the number of IFN-γ-expressing cells was quantified as spot forming cells (SFC) per 5.0×10^4 cells. Values are shown for three separate donors. (C) Comparison of EBV specificity between NT EBVSTs and CD30.CAR-EBVSTs with values calculated to include background subtraction of the "no pepmix" condition (n = 3). (D) Poly-functionality of EBVSTs was assessed using the FluoroSpot assay to measure IFN-γ, granzyme B, and TNF-α. The number of SFC positive for all three molecules per 2.5×10^4 cells was quantified. (E) Diagram illustrating simplified second stimulation protocol using only the ULCL to provide both antigen signal and co-stimulation. (F) Quantification of EBV specificity comparing CD30.CAR EBVST generated with pxATC or without pxATC in the second stimulation (n = 3). Different symbols denote individual donors. Data denoted as mean ± SEM. Co-stim, co-stimulatory.

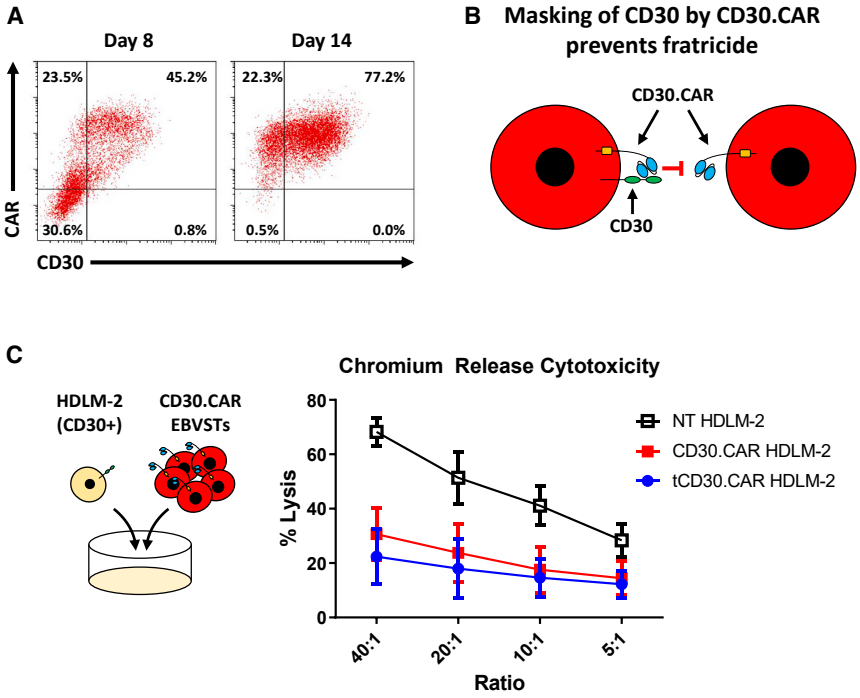


Figure 3. CD30.CAR prevents CD30-mediated fratricide

(A) Representative flow plot showing expression of CD30 and CD30.CAR in CD30.CAR-EBVSTs. (B) Illustration depicting masking of CD30 by a CD30.CAR *in cis*. (C) CD30.CAR-EBVSTS were co-cultured with CD30-positive HDLM-2 tumor cells that were NT or transduced with CD30.CAR or truncated (t) CD30.CAR. Cytotoxicity was measured in a 4- to 5-h chromium release assay. Data denoted as mean ± SEM (n = 3).

when engaging in subsequent encounters. To determine if CD30.CAR-EBVSTs can resist killing by previously primed alloreactive T cells (p-ARTs), we generated EBVST donor-specific p-ARTs by co-culturing PBMCs with irradiated allogeneic PBMCs from the EBVST donor. We then co-cultured donor NT EBVSTs or CD30.CAR-EBVSTs with the p-ARTs at a ratio of 5:1 (Figure 6A). NT EBVSTs were eliminated by p-ARTs while CD30.CAR-EBVSTs resisted killing and proliferated/persisted in the cultures (Figure 6B). In addition, Figure 6C shows p-ART expansion was prevented by CD30.CAR EBVSTs. To assess whether rejection-resistant CD30.CAR-EBVSTs can eliminate tumor cells, we set up a triple co-culture of $CD30^+$ tumor cells, p-ARTs, and CD30.CAR-EBVSTs at a ratio of 2:1:5 (Figure 6D). We used the HLA-negative ULCLs to avoid potential allo-specific killing of tumor cells by the p-ARTs. CD30.CAR-EBVSTs could eliminate $CD30^+$ ULCLs in the presence of p-ARTs, similar to conditions without p-ARTs (Figure 6E).

CD30.CAR-EBVSTs target activated NK cells that express CD30

NK cells also recognize and kill allogeneic donor cells and can contribute to rejection of OTS T cell products via killer-cell immunoglobulin-like receptors (KIRs) binding to mismatched HLA class I molecules.[29,30] To evaluate the kinetics of CD30 expression on NK cells, CD56-selected PBMCs were activated using an HLA-negative cell line K562 genetically modified to express membrane-bound IL-15 and 41BB ligand (K562-mb15-41BBL).[31] CD30 expression was increased from 12.5% ± 15.4% of NK cells on day 0 to more than 95.55% ± 2.65% by day 8 (Figure 7A). To determine if CD30.CAR EBVSTs could eliminate activated NK cells, we set up MLRs at a 5:1 ratio of HLA-A2-positive EBVSTs:HLA-A2-negative NK cells and found that NK cells were significantly decreased when co-cultured with CD30.CAR EBVSTs compared with NT EBVSTs (Figure 7B); those that remained were mostly CD30 negative (Figure 7C).

DISCUSSION

We have evaluated CD30.CAR-expressing EBVSTs as a platform to address the two major challenges of OTS T cell therapy, namely, GVHD and allogeneic graft rejection. Since VSTs rarely cause GVHD when given to allogeneic recipients,[14] we used EBVSTs as our T cell platform and transduced them with a CD30.CAR that could eliminate both tumor and activated alloreactive T cells. Despite their expression of CD30, CD30.CAR-EBVSTs proliferated normally due to *cis* binding of CD30.CAR to CD30, which protected them from fratricide. CD30.CAR-EBVSTs retained function through both the CAR and their native EBV TCRs, and CD30.CAR-EBVSTs could eliminate alloreactive T cells as well as NK cells that express CD30 *in vitro* after activation, providing a mechanism for rejection resistance.

Effector cells other than VSTs lack the capacity for GVHD, either inherently, for example, NK cells, NKT cells, or γδ T cells, or after engineering to remove their endogenous αβ TCRs.[10] VSTs offer several advantages as an OTS platform, including their proven safety in allogeneic recipients, ability to proliferate in patients in response to viral reactivation or vaccination, and their memory potential that allows them to persist long term.[12,32,33] In addition, VSTs can traffic to sites of infection or inflammation and could have advantages in homing to tumors. Indeed, bystander VSTs that lack tumor reactivity have been identified within solid tumor microenvironments, illustrating their ability to traffic to and enter tumor sites.[34,35]

The first OTS T cell therapy used EBVSTs to treat EBV associated post-transplant lymphoproliferative disease and was shown to be both safe and effective in eight patients with three complete remissions and no GVHD.[36] In a follow-up study, none of the 33 patients developed GVHD and 17 patients had tumor responses.[37] The majority of these patients had received solid organ transplants (SOTs) and required continued immunosuppression that likely inhibited both

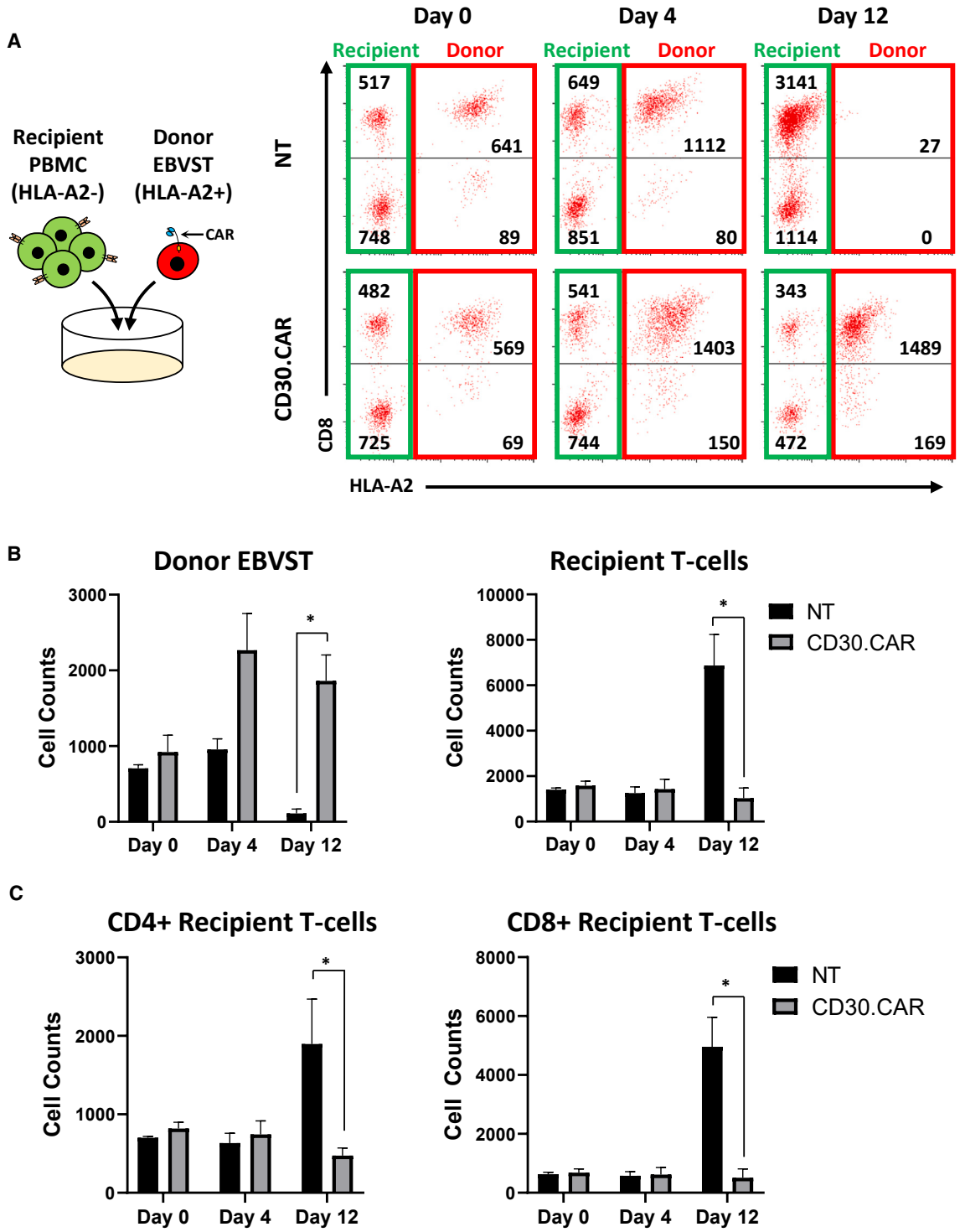


Figure 4. CD30.CAR protects EBVSTs from killing by allogeneic PBMCs

(A) MLRs were established by co-culturing donor EBVSTs (HLA-A2 positive) that were either NT or CD30.CAR transduced with recipient allogeneic PBMC (HLA-A2 negative). Representative dot plots analyzed on day 0, day 4, and day 12 show expression of $CD8^+$ and HLA $A2^+$ on T cells (gated on CD3). Cell counts are quantified with counting beads. (B) Quantification of total $CD3^+$ donor EBVSTs (HLA-A2 positive) and $CD3^+$ recipient allogeneic T cells (HLA-A2 negative) within the co-cultures on the days indicated. (C) Quantification of $CD3^+$ recipient T cells further gated on CD4 or CD8. Data denoted as mean ± SEM (n = 3–4 donor-recipient pairs). Significance was determined using unpaired two-tailed Student's t tests. *$p < 0.05$ when comparing NT and CD30.CAR.

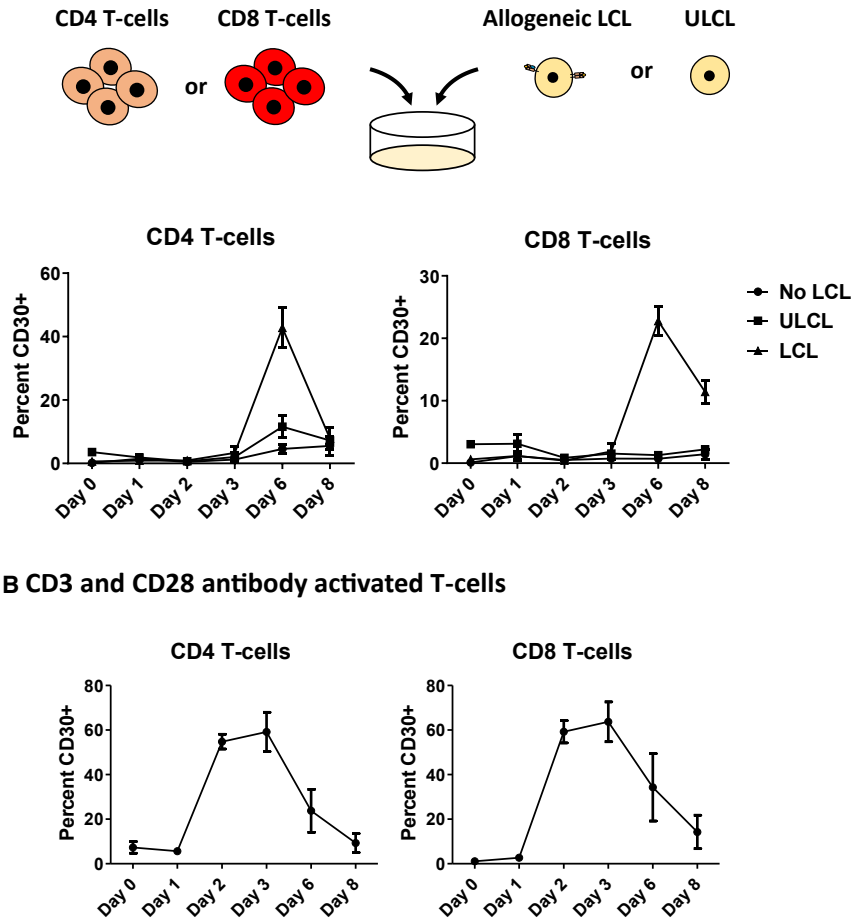


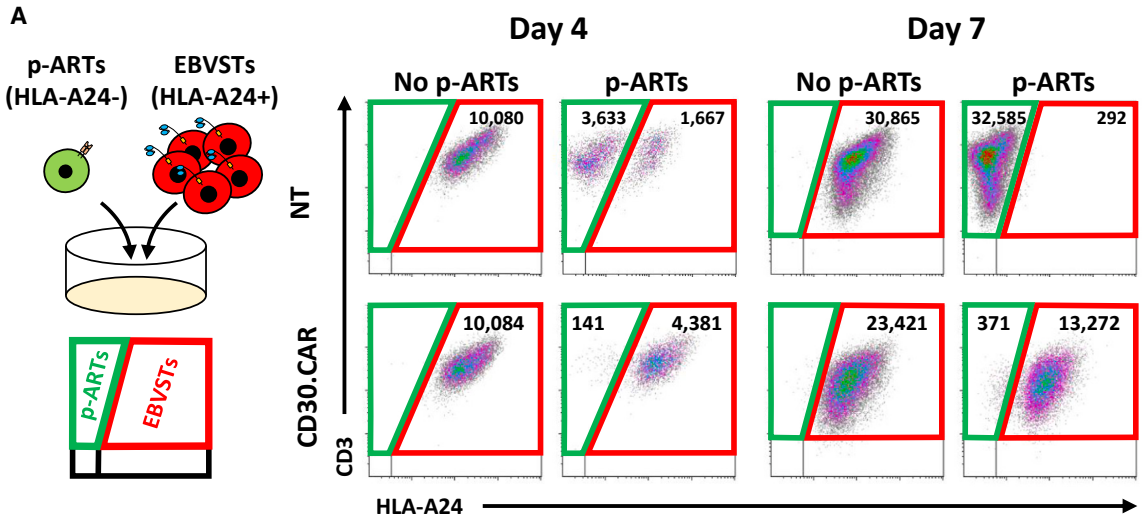
Figure 5. Kinetics of CD30 expression on T cells

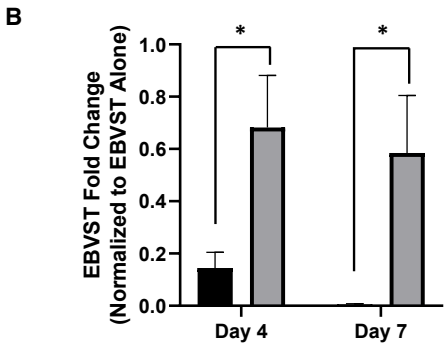
(A) To observe CD30 upregulation on alloreactive T cells, CD4- or CD8-positive cells isolated from PBMCs were co-cultured with irradiated allogeneic LCLs or the HLA-negative ULCL at a ratio of 20:1. The frequency of $CD30^+$ T cells was measured at the time points indicated using flow cytometry. (B) To activate all T cells, CD4- or CD8-positive T cells were isolated from PBMCs and stimulated with plate bound anti-CD3 and anti-CD28. CD30 expression was assessed by flow cytometry. Data denoted as mean ± SEM (n = 3).

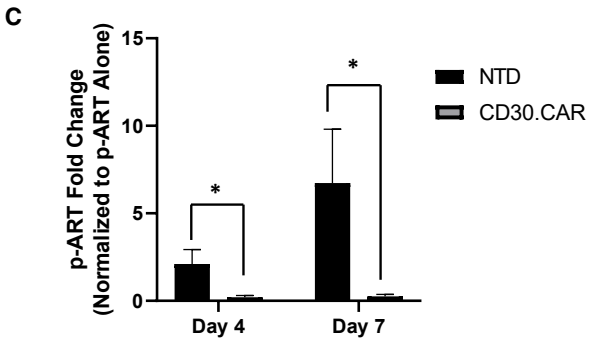
rejection and the function of the infused T cells, so that multiple infusions were required to produce tumor responses. Later studies showed that OTS T cells specific for multiple viruses, including EBV, were even more effective in the HSCT setting likely due to the more profound endogenous immunosuppression of HSCT recipients that limited rejection, and lack of iatrogenic immunosuppression that would inhibit VST function.[16,38] Supporting this hypothesis, Prockop et al.[39] showed that HSCT recipients had higher complete response rates to OTS VSTs than SOT recipients. Outside the transplant setting, rejection is a far greater problem and active steps will likely be required to mitigate it.

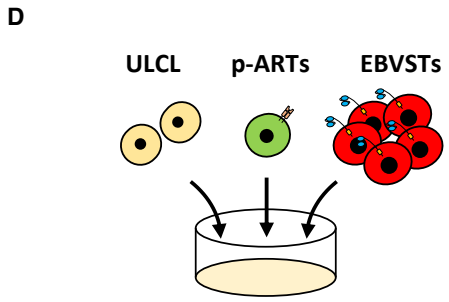
A common strategy to prevent rejection is to eliminate surface expression of HLA class I and II molecules, by knocking out B2M, the universal component of HLA class I molecules[9] and CIITA (class II major histocompatibility complex transactivator), the master transcription factor for HLA class II antigen expression, respectively.[17] Although effective at preventing T cell-mediated rejection, HLA class I loss increases susceptibility to killing by NK cells.[18] To address this problem, non-polymorphic HLA molecules such as HLA-E and HLA-G can be expressed,[40,41] but since NK cells are a heterogeneous population with multiple recognition receptors, the efficacy of this strategy may be limited.[42] An alternative approach to prevent rejection is to directly target alloreactive T cells. In 1980, Miller[43,44] introduced the concept of a "veto cell" that can specifically eliminate cognate alloreactive T cells. This veto effect was independent of TCR ligation and mediated instead by a Fas-FasL dependent mechanism, in which FasL expressed on veto cells binds to Fas on alloreactive T cells, inducing apoptotic cell death.[45–47] However, to date, this strategy has not been validated in the clinic. To generate a veto cell that can eliminate alloreactive T cells via TCR-mediated cytolysis, our lab developed the CHAR molecule and showed CHAR-modified T cells could eliminate alloreactive T cells and persist in MLRs.[19] However, CHAR-modified T cells proliferated poorly due to fratricide, limiting their clinical potential. Extending upon this work, our group has shown that ATCs expressing a 4-1BB.CAR could kill alloreactive T cells, enabling their improved persistence and resulting in better tumor control in mice.[20] In the present study, we targeted the T cell activation marker CD30 both to prevent allo-rejection and mediate the elimination of $CD30^+$ tumors.

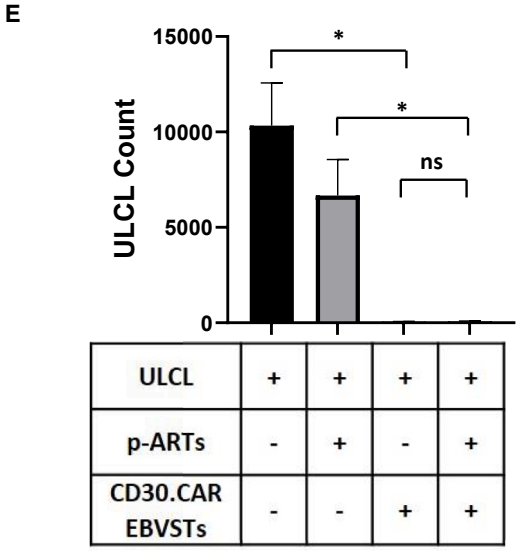
Since ATCs upregulate CD30, it was surprising that CD30.CAR ATCs grew normally with no signs of fratricide.[48] Here, we explain this phenomenon by showing that binding of CD30.CAR to CD30 *in cis* prevents killing *in trans* from other CD30.CAR-EBVSTs. The ability of the CAR to mask its cognate antigen *in cis* has previously been reported by Ruella et al.,[49] who showed that leukemic B cells accidentally transduced with a CD19.CAR during CD19.CAR manufacture were protected from CD19 CAR T cell killing. Understanding this mechanism of fratricide resistance has potential utility in informing the design of CARs targeting T cell malignancies via antigens also expressed on healthy T cells. *Cis* binding of CD30.CAR to CD30 could produce tonic signaling within the cell, and indeed we found











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CD30.CAR-EBVSTs had higher expression of the activation markers CD137 and CD25 and antigen-independent secretion of IFN-γ, granzyme B, and TNF-α. However, despite this increased background signaling, CD30.CAR-EBVSTs showed comparable functionality to NT EBVSTs with maintenance of cytotoxicity against $CD30^+$ targets and robust EBV specificity. Further CD30.CAR-ATCs have not produced severe cytokine-related toxicity in clinical trials.[26]

To simplify and optimize CD30.CAR-EBVST manufacturing for clinical application, we made some changes to the manufacturing protocol used for the generation of EBVSTs and CD30.CAR ATCs. To reduce the high spontaneous cytokine secretion in the ELISpot assay, we evaluated different growth media and found that medium containing HPL produced less background activity. HPL not only has the advantages of being animal free and having reliable lot-to-lot consistency,[50] but was also found to improve the function of CAR T cells.[51] We also showed that TCR stimulation could be replaced by CAR stimulation without loss of antigen specificity, eliminating the need for pxATC during the second stimulation and streamlining the manufacturing process. In addition, since CD30.CAR possesses the dual functions of rejection resistance and anti-tumor activity, only a single transgene is required, further simplifying the protocol and reducing the overall cost.

CD30.CAR-EBVSTs resisted killing by both naive and p-ARTs. Notably, p-ARTs were more potent than naive alloreactive T cells present within PBMCs, since they have acquired effector functions, but CD30.CAR-EBVSTs were able to eliminate them more rapidly since they already expressed CD30. There are several advantages in targeting CD30 to prevent rejection, which includes its expression on both CD4 and CD8 alloreactive T cells, and on NK cells that could mediate rejection through mismatched KIRs.[29,30] We have not directly shown that CD30.CAR-EBVSTs are protected against alloreactive NK cell-mediated rejection, but we found that activated NK cells express CD30 and can be eliminated by CD30.CAR EBVSTs. Since CD30.CAR-EBVSTs are resistant against even primed alloreactive T cells, they could likely also resist NK cell-mediated rejection.

While CD30.CAR-EBVSTs could resist rejection and simultaneously kill $CD30^+$ tumor cells *in vitro*, we were unable to test their function in an *in vivo* mouse model due to the constraints of the CD30.CAR design used both in this study and in our clinical trials. Our CD30.CAR is engineered with an IgG1-derived Fc (CH2-CH3) spacer domain that allowed for direct labeling,[48] but several groups have reported that CAR T cells containing the IgG1 Fc domain become trapped in the lungs of NSG mice due to interactions with local Fc receptor-expressing murine cells.[52–54] This severely impairs trafficking of human CD30.CAR-EBVSTs to tumor sites, limiting anti-tumor activity in mice. We attempted to circumvent this issue by infusing CD30.CAR-EBVSTs into the intraperitoneal cavity of mice, but they were rapidly eliminated, in contrast with NT EBVSTs. This has not been a problem in clinical studies, since our CD30.CAR has produced an overall response rate of 72% in 32 patients, of which 59% were complete.[26] Notably, the CH_2-CH_3 spacer domain may have advantages in terms of clinical safety since CARs containing this domain have produced little cytokine release syndrome compared with other CARs,[25,55] and it has previously been demonstrated that altering the spacer/hinge region and transmembrane domains of the CAR can greatly decrease their toxicity profile in patients.[56]

CD30.CAR-expressing ATCs have already proven safe in patients; however, since CD30 is expressed on all ATCs including VSTs, there is the possibility for on-target off-tumor toxicity to affect endogenous immunity. Fortunately, uncontrolled viral infections or reactivations have not been associated with the use of CD30.CAR T cells,[25] but if CD30.CAR-EBVSTs were to greatly expand in response to alloreactive T cells and persist long term in patients, then any potential toxicities could be mitigated by incorporating an inducible suicide switch to allow the elimination of our cells with the administration of a drug.[57] However, given the potency of the allo-rejection response and the slow upregulation of CD30 in alloreactive T cells, it is also possible that CD30.CAR will be unable to fully prevent elimination of our OTS cells. If so, we could explore other T cell activation markers with more rapid expression kinetics.

CD30.CAR-EBVSTs have the potential to treat not only CD30+ cancers but also EBV-positive malignancies, regardless of whether they express CD30. Further, CD30.CAR-EBVSTs would provide a platform to express CARs or TCRs targeting other malignancies and extend the promise of an OTS approach to a broader array of patients. Since, CD30 is upregulated on activated T cells, CD30.CAR EBVSTs could also treat conditions or diseases associated with overactive T cells such as autoimmunity[58,59] or GVHD. Indeed, it has been reported that patients with acute GVHD had greater frequencies of CD30-expressing $CD8^+$ T cells and higher levels of soluble CD30 in their plasma,[60] and two clinical trials evaluating the effect of brentuximab vedotin, an antibody-drug conjugate targeting CD30, for the treatment of either acute or chronic GVHD have shown clinical responses.[61,62]

Figure 6. CD30.CAR-EBVSTs resist killing by primed alloreactive T cells

(A) MLRs were established by co-culturing HLA-A24 negative primed alloreactive T cells (p-ARTs) with HLA-A24-positive EBVSTs. Representative dot plots analyzed on day 4 and day 7 showing cell counts for conditions with EBVSTs grown without p-ARTs and with p-ARTs. (B) Fold change of EBVSTs after co-culture with p-ARTs normalized to the condition of EBVSTs cultured alone (n = 4 donor-recipient pairs). (C) Fold change of p-ARTs after co-culture with EBVSTs normalized to the condition of p-ARTs cultured alone (n = 4 donor-recipient pairs). (D) Schematic of *in vitro* triple co-culture of ULCL, p-ARTs, and EBVSTs. (E) ULCL counts on day 6 were quantified using counting beads (n = 3 donor-recipient pairs). Data denoted as mean ± SEM. Significance was determined by unpaired two-tailed Student's t test. *$p < 0.05$ when comparing NT and CD30.CAR. p-ARTs, primed alloreactive T cells.

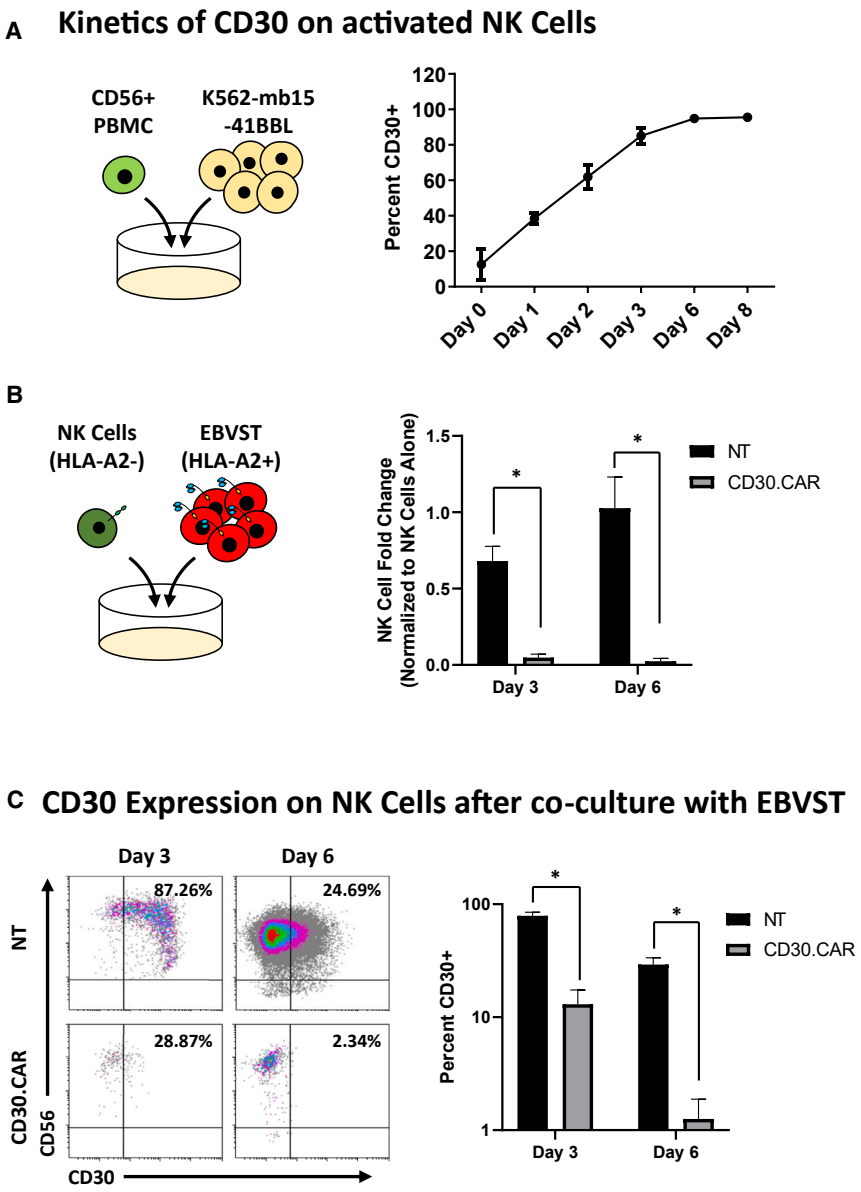

Figure 7. Activated NK cells upregulate CD30 and are eliminated by CD30.CAR-EBVSTs

(A) CD56$^+$ cells were isolated from HLA-A2 negative PBMCs and expanded using K562-mb15-41BBL feeder cells irradiated at 100 Gray (Gy). CD30 expression on CD56$^+$ NK cells was measured by flow cytometry over several days (n = 3). (B) On day 8 of activation, NK cells were co-cultured with HLA-A2-positive NT EBVSTs or CD30.CAR-EBVSTs. NK cell numbers were quantified after co-culture with EBVSTs by calculating the fold change normalized to the condition of NK cells grown alone (n = 5). (C) Representative dot plots (left) and quantification (right) showing CD30 expression on gated NK cells after co-culture with EBVSTs (n = 5). Data denoted as mean ± SEM. Significance was determined by unpaired two-tailed Student's t test. *p < 0.01 when comparing NT and CD30.CAR.

In conclusion, we have addressed the dual challenges of OTS T cell therapy by generating CD30.CAR-modified EBVSTs, which lack host alloreactivity due to their restricted TCR repertoire and can resist rejection by targeting alloreactive T cells. The simplicity and safety of this approach has the advantage of readily translating to the clinic, which we have demonstrated with the timely initiation of a phase I clinical trial (NCT04288726) to assess CD30.CAR EBVSTs as an OTS therapy to treat CD30-positive lymphoma.

MATERIALS AND METHODS

Donors and cell lines

Blood samples were obtained from healthy donors with informed consent in a protocol approved by the Institutional Review Board of Baylor College of Medicine and in accordance with the guidelines established by the Declaration of Helsinki. PBMCs were isolated by density gradient centrifugation using Lymphoprep (STEMCELL Technologies, Vancouver, Canada) according to manufacturer's instructions. The HDLM-2 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The BJAB cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The HLA negative LCL line (ULCL) was generated in our lab as previously described.[27] The genetically modified K562 cell line expressing membrane-bound IL15 and 41BB-ligand (K562-mb15-41BB-L) was a kind gift from Dr. Dario Campana (National University of Singapore).[31] HDLM-2, BJAB, ULCL, and K562-mb15–41BB-L cells were maintained in RPMI 1640 media (Hyclone, Cytiva, Marlborough, MA, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% GlutaMAX (Thermo Fisher Scientific). Cell were grown at 37° in a humidified atmosphere containing 5% carbon dioxide.

Flow cytometry

The following fluorochrome-conjugated monoclonal antibodies were used in this study: CD3 (Clone UCHT1), CD4 (Clone 13B8.2), CD8 (Clone SFCI21Thy2D3), CD19 (J3-119), CD69 (Clone TP1.55.3), and HLA-DR (Clone Immu-357) from Beckman Coulter (Indianapolis, IN, USA). CD30 (Clone BY88), HLA-A2 (Clone BB7.2), HLA-B7 (Clone BB7.1), CD56 (Clone HCD56), PD-1 (Clone NAT105), LAG-3 (Clone 7H2C65), Tim-3 (Clone F38-2E2), CD39 (Clone A1) TIGIT (Clone A15153G) and IgG Fc (Clone M1310G05) from BioLegend (San Diego, CA, USA). CD25 (Clone 2A3), CD134 (Clone

ACT35), CD137 (Clone 4B4-1), from BD Biosciences (Franklin Lakes, NJ, USA). HLA-A24 from LifeSpan Biosciences (Lynnwood, MA, USA). HLA-B8 (Clone REA145) from Miltenyi Biotec (Bergisch Gladbach, Germany). Cells were stained with antibodies for 15–20 min at 4°C. The CD30.CAR was labeled with a human IgG Fc antibody and the cells required at least two washes before staining to remove free IgG present in HPL used in the culture medium. Cell viability was assessed using 7-amino actinomycin D (7-AAD) staining (BD Biosciences). Cell counts were assessed with CountBright Absolute Counting Beads (Thermo Fisher Scientific). We used the Gallios Flow Cytometer (Beckman Coulter) to acquire flow cytometric data and Kaluza Analysis Software (Beckman Coulter) to analyze data and for graphical representation.

Generation of retroviral constructs and retrovirus transduction

The CD30.CAR construct with the HRS3 single chain variable fragment, IgG1-derived Fc (CH2-CH3) spacer domain, and CD28 endodomain used in this study has been previously described.[48] To generate the truncated CD30.CAR, primers were designed to amplify the CD30.CAR construct without the CD3 zeta and CD28 endo-domains and cloned into the gamma retroviral vector SFG using In-Fusion cloning (Takara Bio USA, Mountain View, CA, USA). Transient retroviral supernatants were produced as previously described.[63] To transduce T cells and HDLM-2 cells, retroviral supernatants were added to non-tissue culture treated 24-well plates pre-coated with RetroNectin (Takara Bio, Shiga, Japan). After centrifugation at 2,000×*g* for 90 min, retroviral supernatants were removed and cells were plated in their respective medium, and then transferred to a 37°C, 5% CO_2 incubator.

Generation of ATCs

ATCs were generated by plating PBMCs in 24-well plates coated with 1 mg/mL anti-CD3 (from OKT3 hybridoma cell line ATCC# CRL-8001, Manassas, VA, USA) and 1 mg/mL anti-CD28 (BD Biosciences). ATCs were maintained in CTL medium consisting of a 1:1 mix of Advanced RPMI 1640 Medium (Thermo Fisher Scientific) and Click's Medium (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% GlutaMAX (Thermo Fisher Scientific). Both cytokines IL-7 and IL-15 (R&D Systems, Minneapolis, MN, USA) were added to CTL medium at 10 ng/mL. ATCs were split and fed every 2–3 days with fresh CTL medium supplemented with IL-7 and IL-15.

Generation of primed alloreactive T cells

To generate p-ARTs, PBMCs from the EBVST donor were irradiated at 30 Gray (Gy) using an RS2000 X-ray irradiator (RadSource, Suwanee, GA, USA), then co-cultured at a 1:1 ratio with PBMCs from an HLA-mismatched recipient in CTL medium without cytokines. IL-7 and IL-15 were added on day 1 at 10 ng/mL. On days 7–8, cells were harvested and restimulated by plating on anti-CD3 and anti-CD28 coated plates in CTL medium and cytokines. After another 7–9 days, cells were used in assays or cryopreserved for later use. We fed p-ARTs with fresh CTL medium and cytokines every 2–4 days as needed. Cryopreserved p-ARTs were thawed and rested overnight in CTL medium with IL-7 and IL-15 at 10 ng/mL.

Generation of EBVSTs

EBVSTs were generated as previously described.[27] Briefly, PBMCs were depleted of CD45RA positive cells by magnetic column separation using CD45RA microbeads (Miltenyi Biotec) according to manufacturer's instructions. Depleted PBMCs were stimulated with pepmixes representing EBV antigens EBNA1, LMP1, LMP2, and BZLF1 (JPT Peptide Technologies, Berlin, Germany). EBVSTs were transduced with CD30.CAR on days 5–6 and then re-stimulated on days 9–10 with irradiated pepmix-pulsed ATCs (pxATCs) and irradiated ULCLs at an EBVST:pxATCs:ULCL ratio of 1:1:5. In some experiments, we excluded pxATCs and co-cultured EBVSTs on days 9–10 with ULCL at a 1:5 ratio. On days 16–20, cells were cryopreserved for later use. EBVST were grown in VST medium consisting of a 1:1 mix of Advanced RPMI 1640 Medium (Thermo Fisher Scientific) and Click's Medium (FUJIFILM Irvine Scientific) supplemented with 5% HPL (nLiven PR, Biolife Solutions, Bothell, WA, USA) and 1% GlutaMAX (Thermo Fisher Scientific). Both cytokines IL-7 and IL-15 were added to VST medium at 10 ng/mL. Every 2–3 days, EBVSTs were fed with fresh media containing cytokines. Cryopreserved EBVSTs were thawed and rested overnight in VST medium with IL-7 and IL-15 at 10 ng/mL.

Generation of NK cells and co-culture with EBVSTs

NK cells were generated as previously described with slight modifications.[64] Briefly, $CD56^+$ PBMCs were selected from healthy donors using CD56 microbeads (Miltenyi Biotec) and co-cultured with irradiated (100 Gy) K562-mb15-41BB-L at a 1:10 ratio for 8 days in NK cell medium consisting of Stem Cell Growth Medium (Sartorius CellGenix, Freiburg, Germany) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific). IL-2 (NIH, Bethesda, MD, USA) was added at 500 IU/mL. Activated NK cells were co-cultured with EBVSTs at a 1:5 ratio in VST medium with IL-7 and IL-15 at 10 ng/mL. On the indicated days, cells were harvested, stained with antibodies and analyzed by flow cytometry. Countbright Beads (Life Technologies, Carlsbad, CA, USA) were used to assess cell numbers.

MLR assay of EBVSTs with alloreactive T cells

For unprimed MLRs, EBVSTs and allogeneic PBMCs were co-cultured at a 1:4 ratio in CTL medium with IL-2 at 20 IU/mL. For primed MLRs, EBVSTs and p-ARTs were co-cultured at a 5:1 ratio in CTL medium with IL-7 and IL-15 at 10 ng/mL. For MLRs with ULCL tumor cells (triple co-cultures), the ratio of ULCL:p-ARTs:EBVSTs was 2:1:5. On the indicated days, cells were harvested, stained with antibodies, and analyzed by flow cytometry.

Measuring kinetics of CD30 upregulation on alloreactive T cells, ATCs, and NK cells

To determine the kinetics of CD30 upregulation on alloreactive T cells, CD4- and CD8-positive PBMCs cells were isolated using CD4 and CD8 microbeads (Miltenyi Biotec) and magnetic column

separation. $CD4^+$ and $CD8^+$ cells were co-cultured with irradiated (100 Gy) allogeneic LCLs or ULCLs at a 20:1 ratio in CTL medium without cytokines. For ATCs, PBMCs were stimulated on CD3/28-coated plates in CTL medium without cytokines. For NK cells, $CD56^+$ PBMCs were co-cultured with irradiated (100 Gy) K562-mb15-41BB-L at a 1:10 ratio in NK cell medium with IL-2 at 20 IU/mL. On the indicated days, cells were harvested, stained with antibodies, and analyzed by flow cytometry.

ELISpot analysis

ELISpot analysis for IFN-γ secretion was used to determine responses of EBVST lines to EBV antigens as previously described.[27,28] EBVSTs were plated at 5×10^4 per well in duplicate and incubated overnight with or without 100 ng pepmixes. The frequency of antigen specific responses are expressed as spot-forming cells (SFC) per input cell numbers.

FluoroSpot assay

We used the FluoroSpot Plus Kit to detect baseline expression of human IFN-γ, granzyme B, and TNF-α. (Mabtech, Nacka Strands, Sweden). EBVSTs were plated at either 2.5×10^4 or 5×10^4 per well in duplicate and incubated overnight with or without 100 ng pepmixes. The following day, the plate was developed according to the manufacturer's instructions. The frequency of antigen specific responses are expressed as SFC per input cell numbers. Spots were measured using the Mabtech IRIS reader.

Cytotoxicity assay

The cytotoxic specificity of EBVSTs was measured in a standard chromium-51 (^{51}Cr) release assay. The CD30-negative, EBV-negative BJAB lymphoma cell line and the CD30-positive HDLM-2 Hodgkin's lymphoma cell line were incubated with ^{51}Cr sodium chromate for 1 h then washed and used as targets. NT or CD30.CAR-transduced EBVSTs were used as effectors and were incubated with targets at effector-to-target ratios of 40:1, 20:1, 10:1, 5:1, and 2.5:1 in 96-well plates. After 4–6 h of incubation, supernatants were harvested and ^{51}Cr release was detected with a gamma counter. The percentage of specific lysis was determined from the mean of triplicates as [(experimental release − spontaneous release) ÷ (maximum release − spontaneous release)] × 100.

Statistical analysis

Data are presented as mean ± SEM and statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). Paired two-tailed Student t tests were used for comparisons between two groups. Significance was defined by a p value of less than 0.5.

DATA AND CODE AVAILABILITY

All data in the article and the supplemental information support the findings and conclusions of the study. Raw data and materials generated during the study are available upon reasonable request from the corresponding author.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omton.2024.200814.

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AUTHOR CONTRIBUTIONS

D.H.Q. S.S., and C.M.R. conceptualized the project, designed the study and analyzed the data. H.R.G., Y.D.B., N.N., A.M., and Y.F.H. performed experiments and analyzed data. C.M.R. supervised the study and secured funding. D.H.Q. wrote the initial drafted and C.M.R. reviewed and edited the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

Baylor College of Medicine (D.H.Q. and C.M.R. are listed as inventors) has filed a patent application using the CD30.CAR EBVSTs as an off-the-shelf treatment for cancer. D.H.Q. and C.M.R. received funding from Tessa Therapeutics. C.M.R. has equity in Allovir, Marker Therapeutics, serves on the advisory board of Marker and received royalties from Takeda, Marker, Allovir, and Bellicum. Through her spouse, C.M.R. has conflicts with Allogene, Walking Fish, Turnstone Biologics, Posedia, Tscan, Bluebird Bio, Adaptimmune Therapeutics, Abintus, Onkimmune, Triumvira Immunologics, Memgen LLC, Brooklyn Immunotherapeutics, Coya, nd AstraZeneca Pharmaceuticals.

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