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Original Article

CAR-mediated target recognition limits TCR-mediated target recognition of TCR- and CAR-dual-receptor-edited T cells

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Antigen escape can compromise the efficacy of chimeric antigen receptor- (CAR-) or T cell receptor- (TCR-) engineered T cells. Targeting multiple antigens can effectively limit antigen escape, and combining CAR-with TCR-mediated targeting can significantly broaden the spectrum of targetable antigens. Here, we explored whether dual-antigen specificity can be installed on T cells using combined TCR and CAR engineering to prevent antigen escape of multiple myeloma (MM). We report the generation of CD8 T cells that were transduced to express a transgenic TCR, targeting a peptide derived from transcriptional coactivator BOB1 in the context of HLA-B*07:02, alongside a BCMA-targeting CAR. Those T cells, called TRaCR T cells, efficiently recognized target cells that were resistant to either BOB1 TCR or BCMA CAR T cells, illustrating general dual specificity. In the presence of both antigens, however, target cell recognition was preferentially conferred via the CAR, compromising TCR-mediated target cell recognition. Importantly, this resulted in a survival advantage for tumor cells lacking expression of BCMA in an *in vivo* model of heterogeneous MM. In conclusion, we demonstrate general dual specificity of TRaCR T cells but advise caution when using TRaCR T cells as a strategy to target heterogeneous tumors.

INTRODUCTION

Adoptive transfer of genetically modified T cells has demonstrated curative potential for the treatment of certain cancers.[1] In $CD19^+$ malignancies, treatment with CD19-specific chimeric antigen receptor (CAR)-expressing T cells has resulted in profound responses.[2–5] For multiple myeloma (MM), B cell maturation antigen (BCMA)-targeting CAR T cell therapy has recently been approved for clinical use.[6,7] However, responses are often of limited duration.[8] While the therapy is initially effective, patients can relapse as a result of antigen escape, either due to downmodulation of surface expression of the target antigen or due to complete loss of surface expression, rendering tumor cells untargetable by CAR T cells.[9] For MM, both $BCMA^{low}$ or $BCMA^{negative}$ relapses have been described after BCMA-targeting CAR T cell therapy.[10,11] Additionally, some relapses displayed a normal BCMA expression level, but they revealed mutations in the binding epitope of the respective single-chain variable fragment (scFv), thereby evading BCMA-CAR-mediated recognition.[12] Target cell recognition by BCMA CAR T cells can further be compromised due to the physiological features of BCMA itself. BCMA can be cleaved from the surface of myeloma cells, resulting in the accumulation of soluble BCMA in the plasma of patients.[13] Soluble BCMA can in turn bind to CAR molecules on the surface of BCMA CAR T cells, decreasing their sensitivity.[14,15] Furthermore, BCMA has been described to be trogocytosed by CAR T cells upon target cell engagement.[16] Trogocytosis results in the incorporation of BCMA into the plasma membrane of BCMA CAR T cells themselves, which can result in fratricide and reduced overall performance.[17]

To address these issues, multi-antigen targeting strategies are being developed for the treatment of MM. Multi-antigen targeting approaches typically rely on targeting alternative MM antigens accessible by CARs, but safely targetable MM antigens for CAR therapy are sparsely available. For example, MM-associated surface antigens such as CD138, CD38, or SLAMF7 are also expressed on hematopoietic stem cells or T cells, requiring additional modifications or measures for safe application.[18–20] An alternative MM antigen with an expression profile suitable for CAR T cell therapy is GPRC5D, but targeting GPRC5D faces challenges related to antigen escape as does targeting BCMA.[12,21–23]

Alternatively, T cell receptor (TCR)-engineered T cells can be incorporated into multi-antigen-targeting strategies, as leveraging the combination of human leukocyte antigen (HLA)-dependent and HLA-independent targeting can potentially reduce the occurrence of immune escape.[24] The HLA restriction of TCRs allows for the recognition of a broader antigen pool that includes intracellular and secreted antigens that are usually inaccessible by CAR-mediated targeting.[25] We have previously described TCRs targeting peptides derived from the transcriptional coactivator BOB1 (alternative names

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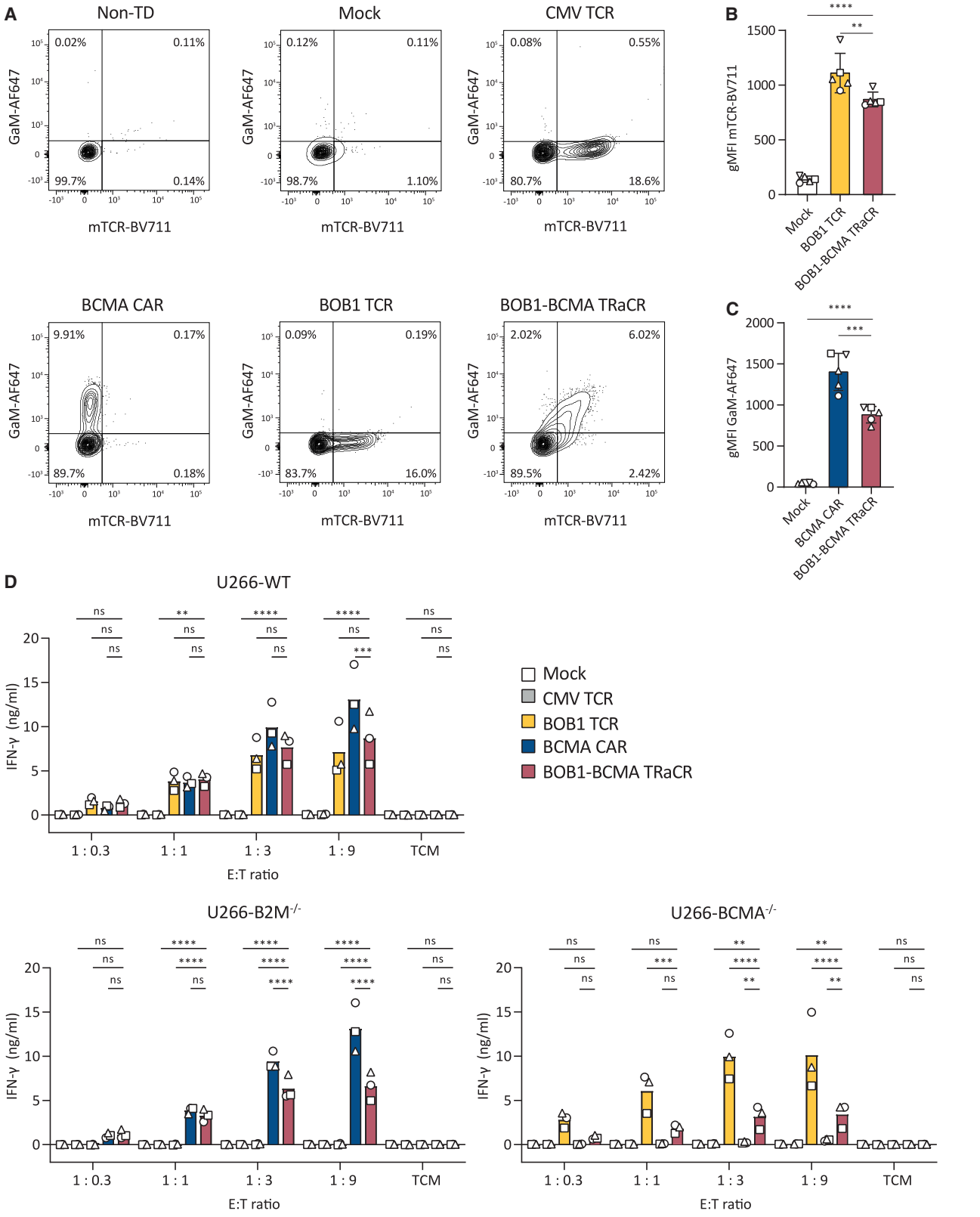

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POU2AF1 and OCA-B).[25,26] BOB1 has a B cell- and plasma cell-restricted expression pattern, where it is essential for proper B cell and plasma cell differentiation and function.[27–29] Importantly, BOB1 has been suggested to be essential for the survival of MM cells and therefore constitutes an attractive immunotherapeutic target.[30–32]

From a manufacturing point of view, it might be attractive to generate a single T cell product that simultaneously expresses both a tumor-targeting TCR as well as a CAR, thereby conferring dual-antigen specificity. Here, we explored both the feasibility of generating T cells that express a transgenic TCR alongside a CAR (called TRaCR T cells), as well as the functional capabilities of those cells in targeting immune escaped MM. Finally, we compared the activity of TRaCR T cells to separately generated engineered TCR (eTCR) or CAR T cells in an *in vivo* model of heterogeneous MM prone to relapse after BCMA CAR T therapy.

RESULTS

Generation of TRaCR T cells

To generate TRaCR T cells that express a transgenic TCR and a CAR at the same time, we cloned a single expression vector encoding an HLA-B*07:02 restricted murinized BOB1 TCR, followed by an internal ribosome entry site preceding a second-generation BCMA-targeting CAR (Figure S1A).[26] CD8 T cells were isolated from healthy donor peripheral blood mononuclear cells (PBMCs) and transduced on day 2 after activation to express either the BCMA CAR alone, the BOB1 TCR alone, or both encoded by the single expression vector. Cytomegalovirus (CMV) TCR and Mock (dNGFR as marker gene only) were included as controls. TRaCR T cells could successfully be generated as indicated by correlating expression of both the introduced BOB1 TCR and the BCMA-targeting CAR (Figure 1A). The transduction efficiency as well as the expression of both receptors was lower than single transduction, probably due to the increased size of the insert (3.5 kb of the double construct vs. 2 kb) (Figures 1B and 1C).

To test TRaCR T cell functionality, transduced T cells were enriched by magnetic-activated cell sorting (MACS) purification for either dNGFR or murine TCR (mTCR) expression. TRaCR T cells were enriched for mTCR expression (Figures S1B and S1C). To test whether TRaCR T cells are capable of recognizing tumor cells via either receptor, purified T cell products were subjected to CRISPR-Cas9-edited derivatives of the MM cell line U266. U266 naturally expresses both the CAR antigen BCMA and HLA-B*07:02 and BOB1. When incubated with U266-wild type (WT) overnight, BOB1 TCR T cells, BCMA CAR T cells, and TRaCR T cells secreted interferon-γ (IFN-γ) in a dose-dependent manner (Figure 1D). When incubated with U266-B2M$^{-/-}$ that completely lacks HLA class I surface expression, BOB1 TCR T cells showed no cytokine secretion, whereas recognition by BCMA CAR T cells was unimpaired. TRaCR T cells recognized U266-B2M$^{-/-}$, indicating that TRaCR T cells can recognize target cells through the introduced CAR. In the effector-to-target ratios (E:T) of 1:3 and 1:9, recognition by BCMA CAR T cells of U266-B2M$^{-/-}$ was superior to TRaCR T cells, possibly as a consequence of lower CAR expression (Figure 1C). Conversely, when incubated with U266-BCMA$^{-/-}$, BCMA CAR T cells did not produce any IFN-γ, while both BOB1 TCR T cells and TRaCR T cells produced cytokines, indicating that TRaCR T cells are able to recognize target cells also through the introduced transgenic TCR. Cytokine secretion of TRaCR T cells that were stimulated through their TCR was lower as compared to BOB1 TCR T cells, again possibly as a consequence of reduced surface expression of the transgenic TCR (Figure 1B). These results demonstrate general dual specificity of TRaCR T cells. However, recognition of single-antigen-expressing target cells through the eTCR or the CAR alone by TRaCR T cells was lower than T cells that were edited to only express one transgenic receptor, likely as a consequence of reduced expression levels from the dual-receptor expression vector.

TRaCR T cells can clear an excess of tumor cells *in vitro*

Some CAR T cells have been described to be sensitive to dysfunction, both antigen independently as a consequence of tonic signaling and antigen dependently as a consequence of exposure to high amounts of antigen.[33] We reasoned that TRaCR T cells could be especially sensitive to overactivation, as they could principally be stimulated simultaneously via both the CAR and the eTCR, leading to increased input signal.

To assess whether TRaCR T cells display signs of antigen-independent or antigen-induced dysfunctionality, we incubated modified T cells with increasing amounts of non-irradiated target cells up to an E:T ratio of 1:9 and tested their long-term tumor clearance, expansion capability, and changes in phenotype. After 6 days of coculture, TRaCR T cells demonstrated near-complete clearance of U266-WT in an E:T ratio of 1:9, comparable to BOB1 TCR T cells or BCMA CAR T cells (Figure 2A). When stimulated with U266-B2M$^{-/-}$ or with U266-BCMA$^{-/-}$, TRaCR T cells also completely cleared tumor cells, whereas single-specific T cell products BOB1 TCR or BCMA CAR could not clear antigen-negative target cells U266-B2M$^{-/-}$ or

Figure 1. Generation of TRaCR T cells

(A) Representative FACS plots showing expression of transgenes on day 5 after retroviral transduction of primary human CD8 T cells. (B) Expression of mTCR on transduced BOB1 TCR or TRaCR T cells. $n = 5$ individual transductions on 4 independent donors (indicated by symbols). Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test. (C) Expression of CAR (goat-anti-mouse) on transduced BCMA CAR or TRaCR T cells. $n = 5$ individual transductions on 4 independent donors (indicated by symbols). Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test. (D) IFN-γ secretion of purified T cell populations after overnight coculture with indicated target cells. Data depict averaged triplicate values from IFN-γ ELISA of supernatants of three individual experiments using T cells derived from independent donors ($n = 3$). Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test comparing TRaCR T cells to other groups.

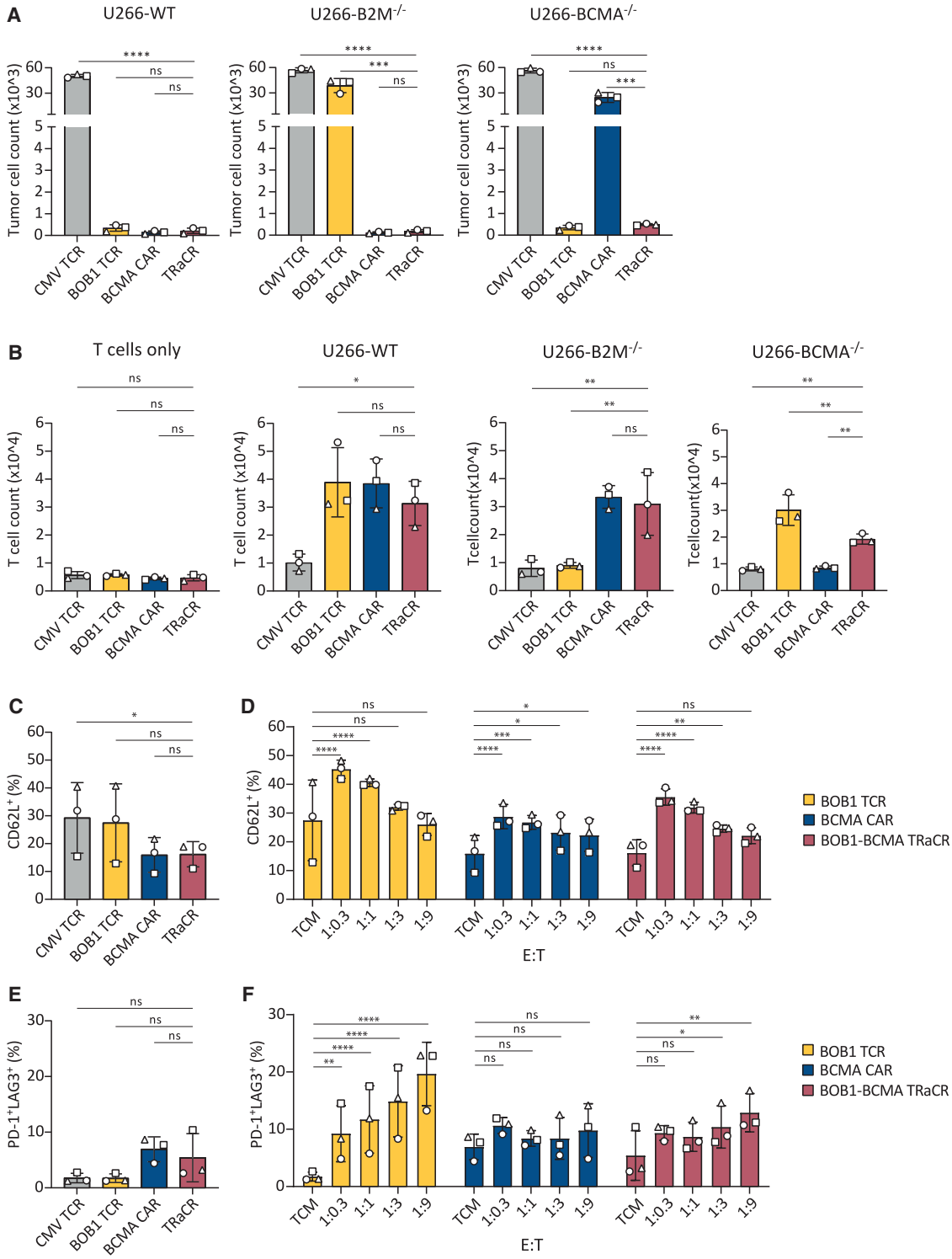

Figure 2. TRaCR T cells can clear an excess of tumor cells *in vitro*

Purified T cells were incubated with U266-WT, U266-B2M$^{-/-}$, or U266-BCMA$^{-/-}$ in E:T ratios of up to 1:9 and incubated for 6 consecutive days. (A) Quantification of viable tumor cells after 6 days of coculture in an E:T ratio of 1:9. Compiled data derived from individually generated T cell products derived from three individual donors. (B) Quantification of T cells after 6 days of culture in the absence (left) or in the presence of indicated tumor cells in an E:T ratio of 1:1. Data depict averaged duplicate values,

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U266-BCMA$^{-/-}$, respectively (Figure 2A). These findings highlight that TRaCR T cells can clear large amounts of tumor cells *in vitro*, irrespective of whether they are stimulated through their eTCR or CAR. In the presence of both antigens when encountering U266-WT, TRaCR T cells similarly cleared all tumor cells, suggesting that the presence of both antigens does not result in detrimental overactivation that would impair tumor cell clearance.

To assess antigen-specific T cell expansion, we quantified the amount of T cells at the end of the 6-day coculture in an E:T ratio of 1:1. CMV TCR T cells, BOB1 TCR T cells, BCMA CAR T cells, and TRaCR T cells yielded comparable numbers in the absence of stimulator cells, suggesting the absence of a strong tonic signal in CAR-expressing cells that would induce antigen-independent proliferation (Figure 2B).[34] After antigen-specific stimulation, TRaCR T cells proliferated when encountering U266-WT, but also after coculture with single-antigen-positive U266-B2M$^{-/-}$ or U266-BCMA$^{-/-}$ (Figure 2B). In contrast, BOB1 TCR T cells did not expand after encountering U266-B2M$^{-/-}$, while BCMA CAR T cells did not proliferate in the presence of U266-BCMA$^{-/-}$. Expansion of BOB1 TCR T cells was more efficient compared to TRaCR T cells when encountering U266-BCMA$^{-/-}$, possibly as a consequence of reduced transgenic TCR expression in TRaCR T cells resulting in suboptimal stimulation. In the presence of the CAR antigen, no difference was observed between TRaCR T cell or BCMA CAR T cell expansion, irrespective of whether HLA was expressed by the target cells.

Furthermore, we assessed changes in the expression of the memory marker CD62L, as well as in the frequency of cells simultaneously expressing the co-inhibitory markers PD-1 and LAG3. Without antigenic stimulation, the frequency of CD62L-expressing TRaCR T cells decreased when compared to CMV TCR or BOB1 TCR T cells, comparable to that of BCMA CAR T cells (Figure 2C). After exposure to low amounts of target cells, the frequency of CD62L-expressing cells increased in BOB1 TCR T cells, BCMA CAR T cells, and TRaCR T cells, while further increase in target cell amounts negatively correlated with frequencies of CD62L^{+} cells in all T cell populations tested (Figure 2D).

Conversely, the frequency of PD-1^{+}LAG3^{+} double-positive T cells showed a tendency to be increased on TRaCR T cells compared to CMV TCR or BOB1 TCR T cells in the absence of target cells, again comparable to that of BCMA CAR T cells (Figure 2E). We reason that the differences in phenotypes without antigenic stimulation may be a consequence of BCMA-CAR-mediated tonic signaling that appears to be comparable between BCMA CAR T cells and TRaCR T cells. The frequency of PD-1^{+}LAG3^{+} T cells correlated positively with target cell exposure in BOB1 TCR T cells (Figure 2F), while for TRaCR T cells, the frequency was further increased in settings of higher target cell exposure. For BCMA CAR T cells, the frequency of PD-1^{+}LAG3^{+} T cells remained largely unchanged.

We conclude that TRaCR T cells can clear large amounts of tumor cells *in vitro* and do not display signs of increased antigen-dependent or antigen-independent dysfunction. Proliferation of TRaCR T cells after stimulation through only the TCR was inferior compared to that of BOB1 TCR. Phenotypically, TRaCR T cells largely resembled BCMA CAR T cells in the absence of antigenic stimulation.

TRaCR T cells preferentially recognize CAR-antigen-expressing target cells, impairing TCR-mediated target cell lysis

We established that TRaCR T cells can be generated and possess general dual-antigen specificity when exposed to isolated single-antigen-expressing cells. We next wondered how target cell recognition of TRaCR T cells is conferred when both antigens are present at the same time. As downstream signaling events of TCRs and CARs largely overlap, it is inherently difficult to dissect whether a TRaCR T cell will recognize a target cell through its TCR or CAR, or both. We therefore designed a preferential target cell recognition assay, where CAR- or TCR-mediated recognition is measured as killing of tumor cells that can only be recognized by either the CAR or the TCR. To this end, we incubated a fixed amount of single-antigen-expressing U266-HLA$^{-/-}$-BCMA$^{+/+}$ or U266-HLA$^{+/+}$-BCMA$^{-/-}$ in a 1:1 ratio with T cells, and we titrated in an increasing amount of target cells that express the other antigen. We then quantified survival of the fixed amount of tumor cells after overnight coculture to assess the degree of "hot target" inhibition as a measure of preferential target recognition.

We assessed whether target cell recognition of U266-HLA$^{-/-}$-BCMA$^{+/+}$ single-antigen-positive target cells is affected by the presence of an excess of U266-HLA$^{+/+}$-BCMA$^{-/-}$ (Figure 3A). When incubated with BCMA CAR T cells or TRaCR T cells, U266-HLA$^{-/-}$-BCMA$^{+/+}$ were completely cleared after overnight coculture in the absence of additional U266-HLA$^{+/+}$-BCMA$^{-/-}$ (Figure 3A). For BCMA CAR T cells, adding in an up to 9-fold excess of U266-HLA$^{+/+}$-BCMA$^{-/-}$ did not result in diminished clearance of tumor cells expressing only the CAR antigen. Similarly, TRaCR T cells mediated complete killing of U266-HLA$^{-/-}$-BCMA$^{+/+}$, even in the presence of a 9-fold excess of target cells expressing the TCR antigen only (Figures 3A and 3B). This indicates that TRaCR T cells can effectively clear CAR-antigen-expressing target cells in the presence of an abundance of bystander cells that express the respective TCR antigen.

Conversely, we assessed whether target cell recognition of U266-HLA$^{+/+}$-BCMA$^{-/-}$ would be affected by the presence of an excess of

pooled from T cell products derived from individual donors. (C) Frequency of CD62L expressing T cells after 6 days of culture in the absence of target cells. (D) Frequency of CD62L-expressing T cells after 6 days of coculture with U266-WT in indicated E:T ratios. Repeated-measures two-way ANOVA paired for donors comparing TCM to other E:T ratios. (E) Frequency of PD-1^{+}LAG3^{+}-expressing T cells after 6 days of culture in the absence of target cells. (F) Frequency of PD-1^{+}LAG3^{+}-expressing T cells after 6 days of coculture with U266-WT in indicated E:T ratios. Repeated-measures two-way ANOVA paired for donors comparing TCM to other E:T ratios. (A)–(C) and (E) Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test comparing TRaCR T cells to other groups.

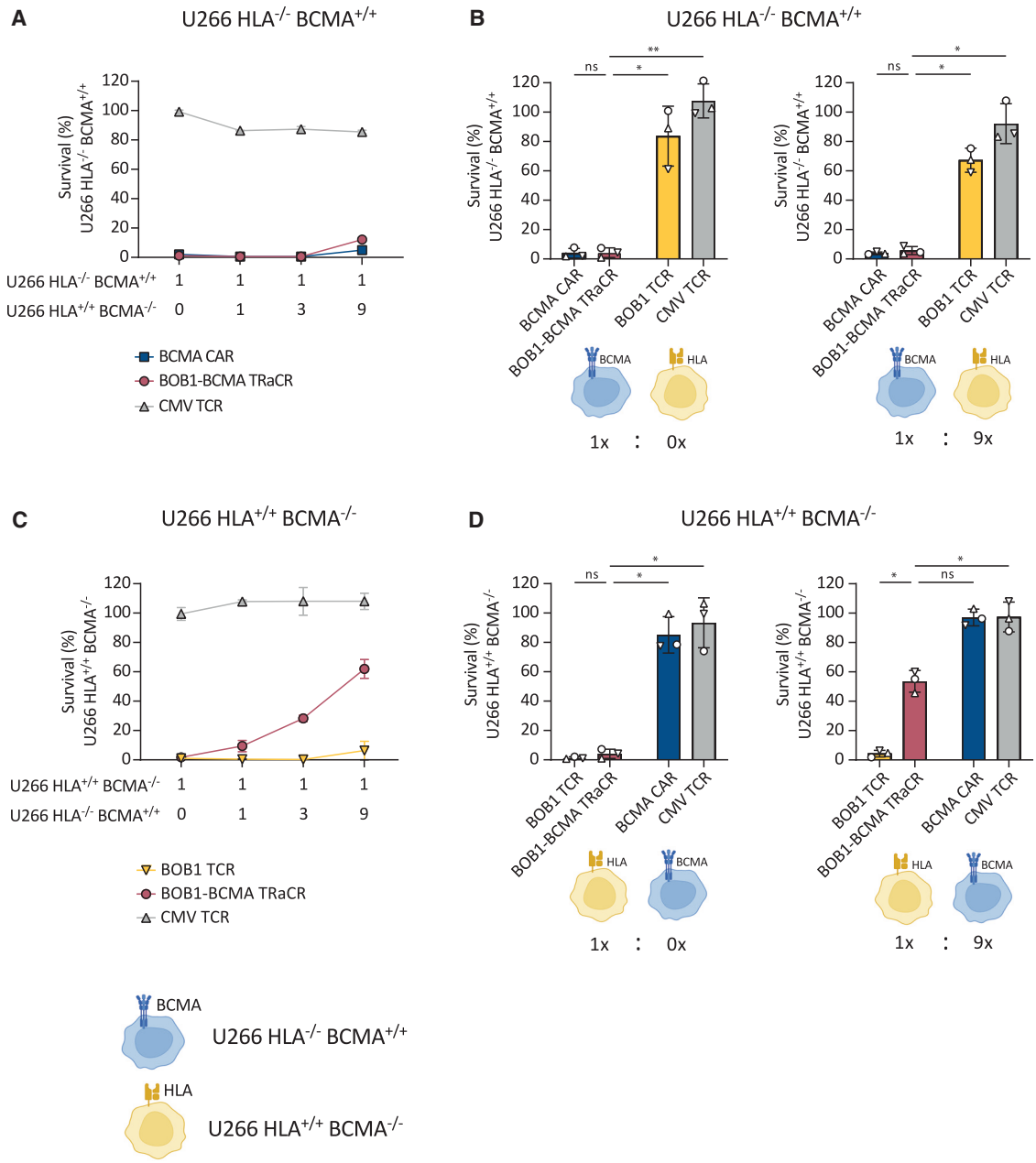
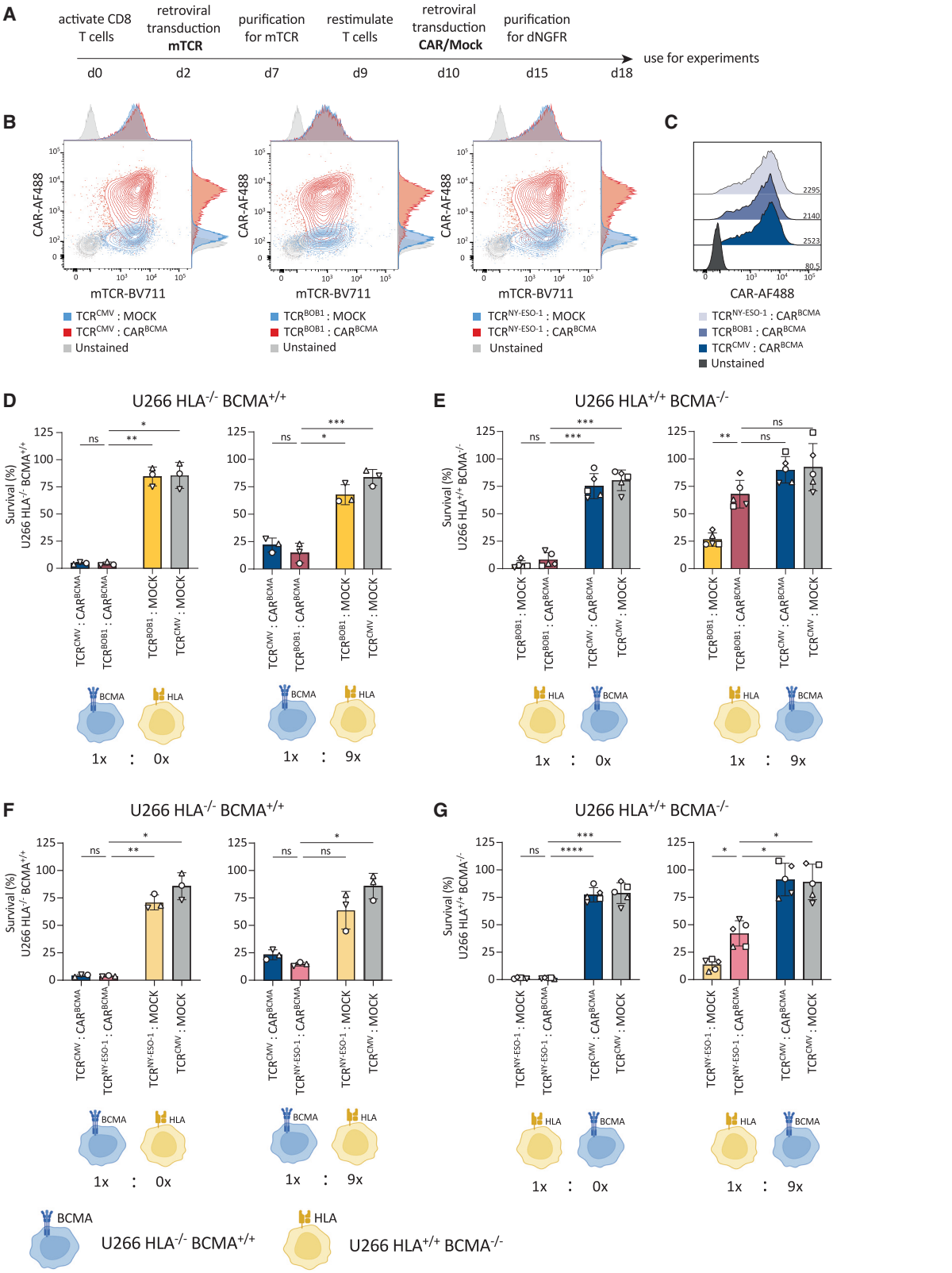


Figure 3. TRaCR T cells preferentially recognize CAR-antigen-expressing target cells, impairing TCR-mediated target cell lysis

(A) Preferential target recognition assay for BCMA-CAR-mediated target recognition. Indicated purified T cell products were incubated with U266-HLA$^{-/-}$-BCMA$^{+/+}$ in a 1:1 ratio, and U266-HLA$^{+/+}$-BCMA$^{-/-}$ were added in the indicated relative frequencies. Survival of U266-HLA$^{-/-}$-BCMA$^{+/+}$ was quantified using flow cytometry after overnight coculture. Data depict frequency of surviving U266-HLA$^{-/-}$-BCMA$^{+/+}$ normalized to tumor cells only in technical duplicates from a representative experiment. (B) Survival of U266-HLA$^{-/-}$-BCMA$^{+/+}$ in the absence (left) or in the presence (right) of a 9-fold excess of U266-HLA$^{+/+}$-BCMA$^{-/-}$. Summary figure generated from three individually generated T cell products. Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test comparing TRaCR T cells to other groups. (C) Preferential target recognition assay for BOB1-TCR-mediated target recognition. Indicated purified T cell products were incubated with HLA$^{+/+}$-BCMA$^{-/-}$ in a 1:1 ratio, and U266-HLA$^{-/-}$-BCMA$^{+/+}$ were added in the indicated relative frequencies. Survival of HLA$^{+/+}$-BCMA$^{-/-}$ was quantified using flow cytometry after overnight coculture. Data depict frequency of surviving HLA$^{+/+}$-BCMA$^{-/-}$ normalized to tumor cells only in technical duplicates from a representative experiment. (D) Survival of HLA$^{+/+}$-BCMA$^{-/-}$ in the absence (left) or in the presence (right) of a 9-fold excess of U266-HLA$^{-/-}$-BCMA$^{+/+}$. Summary figure generated from three individually generated T cell products. Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test comparing TRaCR T cells to other groups.



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U266-HLA$^{-/-}$-BCMA$^{+/+}$ (Figure 3C). Exposure of U266-HLA$^{+/+}$-BCMA$^{-/-}$ to both BOB1 TCR T cells and TRaCR T cells resulted in complete tumor clearance after overnight coculture when there were no additional tumor cells present. When titrating in increasing amounts of CAR-antigen-positive U266-HLA$^{-/-}$-BCMA$^{+/+}$, BOB1 TCR T cell-mediated clearance of TCR-antigen-positive U266-HLA$^{+/+}$-BCMA$^{-/-}$ remained unaffected, maintaining complete tumor clearance irrespective of the amount of titrated U266-HLA$^{-/-}$-BCMA$^{+/+}$. For TRaCR T cells, however, clearance of U266-HLA$^{+/+}$-BCMA$^{-/-}$ was clearly inhibited in the presence of CAR-antigen-positive U266-HLA$^{-/-}$-BCMA$^{+/+}$. Inhibition of TRaCR-TCR-mediated target cell recognition correlated with the increasing presence of CAR-expressing bystander cells, resulting in an approximately 50% reduction in clearance of CAR-antigen-negative target cells (Figures 3C and 3D). We conclude that TRaCR T cells preferentially recognized target cells that express the CAR antigen, resulting in a survival benefit of target cells expressing only the TCR antigen when encountering mixed tumor cells.

Preferential target cell recognition through the CAR is not rescued by improved expression of the TCR and is extendable to other TCR-BCMA-TRaCR combinations

TRaCR T cells preferentially recognized target cells through the CAR, seemingly inhibiting TCR-dependent lysis of tumor cells. Given the observation that mTCR expression was lower in TRaCR T cells as compared to eTCR T cells transduced to only express the BOB1 TCR, we considered that this may be an explanation for suboptimal antigen recognition of TRaCR T cells through the BOB1 TCR during the preferential target cell recognition test. We therefore applied an alternative protocol to generate TRaCR T cells where we could ensure similar expression of the transgenic TCR in both TRaCR T cells and eTCR T cells (Figure 4A). CD8 T cells were first transduced and enriched for mTCR expression. Purified mTCR-expressing T cells were then re-stimulated and transduced a second time to express either the BCMA-targeting CAR or Mock. Retransduced T cells were then MACS purified for dNGFR expression to enrich for CAR or Mock expression, respectively. TCR:CAR TRaCR T cells generated this way showed comparable expression levels of the introduced TCRs, comparable to that of TCR:Mock T cells (Figure 4B). TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells were used as controls. Furthermore, TCR$^{NY\text{-}ESO\text{-}1}$:Mock and TCR$^{NY\text{-}ESO\text{-}1}$:CAR^{BCMA} were included as additional specificities. Between different TCR specificities, CAR expression was also comparable (Figure 4C).

We then used those TRaCR T cells in a preferential target cell recognition assay as described in Figure 3. Again, recognition of U266-HLA$^{-/-}$-BCMA$^{+/+}$ by TCR^{BOB1}:CAR^{BCMA} TRaCR T cells was not affected by the presence of an excess of U266-HLA$^{+/+}$-BCMA$^{-/-}$ (Figure 4D). However, we again observed that TCR-mediated recognition of U266-HLA$^{+/+}$-BCMA$^{-/-}$ by TCR^{BOB1}:CAR^{BCMA} TRaCR T cells was strongly diminished in the presence of a 9-fold excess of U266-HLA$^{-/-}$-BCMA$^{+/+}$ (Figure 4E). This suggests that the expression level of the BOB1 TCR might not be the determining factor of the preferential target recognition phenotype of TRaCR T cells.

We then considered that this observation might be a feature specific for the BOB1 TCR. We therefore continued with TRaCR T cells that were engineered to express a clinically validated TCR targeting an NY-ESO-1-derived peptide in the context of HLA-A*02:01.[35] NY-ESO-1 and HLA-A*02:01 are both naturally expressed by U266. Similar to our previous experience, TCR$^{NY\text{-}ESO\text{-}1}$: CAR^{BCMA} TRaCR T cells displayed reduced recognition of U266-HLA$^{+/+}$-BCMA$^{-/-}$ in the presence of an excess of U266-HLA$^{-/-}$-BCMA$^{+/+}$, whereas recognition of U266-HLA$^{-/-}$-BCMA$^{+/+}$ remained unaffected by the presence of an excess of U266-HLA$^{+/+}$-BCMA$^{-/-}$ (Figures 4F and 4G). These results suggest that preferential target cell recognition of TRaCR by the expressed CAR might be a generalizable feature that is not solely dictated by expression levels and extendable to other TCR-BCMA CAR combinations.

Preferential target cell recognition through the CAR is a function of antigen abundance

After ruling out that the preferential target recognition is a function of differential receptor expression levels between eTCR or TRaCR T cells, we wondered whether preferential antigen recognition would be a consequence of antigen abundance. It is likely that individual stimulatory peptide major histocompatibility complexes (pMHCs) on a cell are massively outnumbered by the number of stimulatory CAR antigens such as BCMA on an MM cell. We therefore devised a system where we could titrate the amount of cognate peptide for TCR-mediated recognition on U266. To this end, we compared the activity of eTCR or TRaCR T cells expressing a virus-specific TCR recognizing the CMV-pp65-derived peptide NLVPMVATV (NLV) in the context of HLA-A*02:01. U266 naturally expresses HLA-A*02:01, whereas CMV-pp65 is not expressed. We titrated pp65 NLV peptide on either U266-HLA$^{+/+}$-BCMA$^{-/-}$ or U266-HLA$^{+/+}$-BCMA$^{+/+}$ (U266-WT) and compared the antigen-specific cytokine

Figure 4. Preferential target cell recognition through the CAR is not rescued by improved expression of the TCR and is extendable to other TCR-BCMA-TRaCR combinations

(A) Timeline for generation of TRaCR T cells by re-stimulation and secondary transduction. (B) Representative FACS plots showing mTCR and CAR expression of TRaCR T cell products generated through re-stimulation and secondary transduction. (C) Overlay of CAR expression of TRaCR products as depicted in (A). (D) Preferential antigen recognition test assessing CAR-mediated target cell recognition of TCR^{BOB1}:CAR^{BCMA} TRaCR T cells generated by re-stimulation as depicted in (A). Summary figure generated from three individually generated T cell products. (E) Preferential antigen recognition test assessing TCR-mediated target cell recognition of TCR^{BOB1}:CAR^{BCMA} TRaCR T cells generated by re-stimulation as depicted in (A). Summary figure generated from five individually generated T cell products. (F) Preferential antigen recognition test assessing CAR-mediated target cell recognition of TCR$^{NY-ESO-1}$:CAR^{BCMA} TRaCR T cells generated by re-stimulation as depicted in (A). Summary figure generated from three individually generated T cell products. (G) Preferential antigen recognition test assessing TCR-mediated target cell recognition of TCR$^{NY\text{-}ESO\text{-}1}$:CAR^{BCMA} TRaCR T cells generated by re-stimulation as depicted in (A). Summary figure generated from five individually generated T cell products. (D–G) Repeated-measures ANOVA paired for donors with Dunnett's multiple comparisons post hoc test comparing TCR:CAR^{BCMA} TRaCR T cells to other groups.

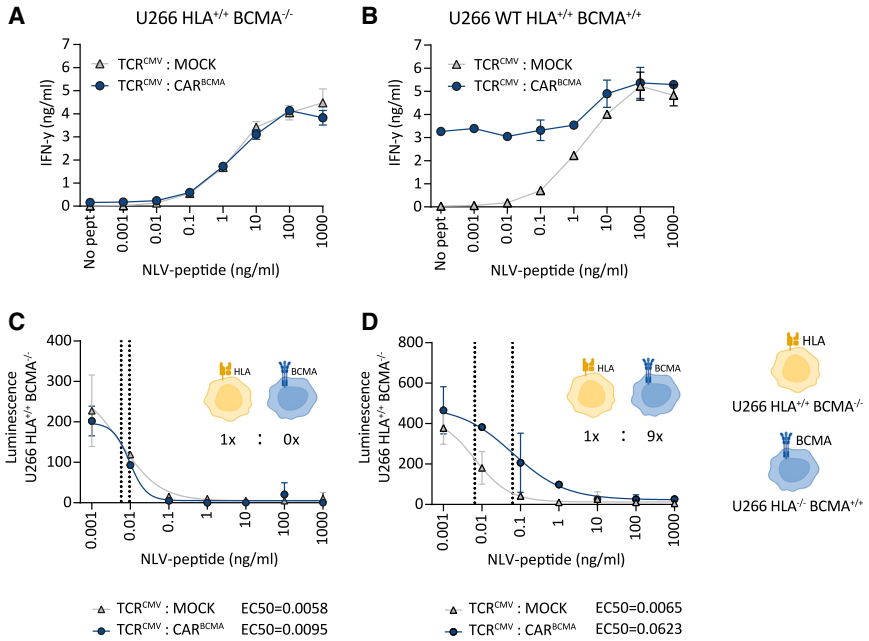

Figure 5. Preferential target cell recognition through the CAR is a function of antigen abundance

(A) TCR-mediated IFN-γ secretion by TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells in response to NLV peptide pulsed on U266-$HLA^{+/+}$-$BCMA^{-/-}$. (B) TCR-mediated IFN-γ secretion by TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells in response to NLV peptide pulsed on U266-$HLA^{+/+}$-$BCMA^{+/+}$ (U266-WT). (C) Viability of NLV peptide-pulsed U266-$HLA^{+/+}$-$BCMA^{-/-}$ after overnight coculture with TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells measured by *in vitro* bioluminescence. Dotted lines indicate half-maximal killing (EC_{50}). (D) Viability of NLV peptide-pulsed U266-$HLA^{+/+}$-$BCMA^{-/-}$ after overnight coculture with TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells measured by *in vitro* bioluminescence in the presence of a 9-fold excess of $HLA^{-/-}$-$BCMA^{+/+}$. Dotted lines indicate EC_{50}. (A–D) Averaged technical duplicates from a representative experiment.

secretion of TCR^{CMV}:Mock T cells to that of TCR^{CMV}:CAR^{BCMA} TRaCR cells (Figure 5A). Both TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells responded identically to increasing amounts of stimulatory peptide pulsed on U266-$HLA^{+/+}$-$BCMA^{-/-}$, demonstrating that the TCR-mediated antigen recognition is generally unaffected in TRaCR T cells compared with eTCR T cells in the absence of the CAR antigen.

After stimulation with U266-$HLA^{+/+}$-$BCMA^{+/+}$ (U266-WT), TCR^{CMV}:Mock T cells responded comparably to increasing amounts of peptide as when stimulated by U266-$HLA^{+/+}$-$BCMA^{-/-}$, secreting detectable amounts of IFN-γ starting from a concentration on of 0.1 ng/mL NLV peptide. TCR^{CMV}:CAR^{BCMA} TRaCR T cells already secreted cytokines in the absence of NLV peptide, which is consistent with BCMA-dependent stimulation via the CAR (Figure 5B). IFN-γ secretion by TCR^{CMV}:CAR^{BCMA} TRaCR cells remained largely unaffected by increasing NLV concentrations up to a concentration of 1 ng/mL. When pulsed with concentrations of NLV peptide of 10 ng/mL, IFN-γ secretion by TCR^{CMV}:CAR^{BCMA} TRaCR cells did further increase and reached a higher level than TCR^{CMV}:Mock T cells, suggesting an additive effect of combined stimulation via BCMA and the TCR antigen at this concentration range. When pulsed with even higher concentrations of 100 ng/mL and 1,000 ng/mL of peptide, IFN-γ secretion reached a plateau for both TCR^{CMV}:Mock T and TCR^{CMV}:CAR^{BCMA} T cells, indicating comparable maximal activation of both TCR^{CMV}:Mock T and TCR^{CMV}:CAR^{BCMA} T cells, even in the presence of BCMA.

We observed an additive effect of combined CAR and TCR stimulation at a concentration of 10 ng/mL NLV peptide, but not at lower concentrations. These results suggest that TRaCR T cells can still respond to TCR antigen when simultaneously stimulated by the CAR, but that their sensitivity toward TCR antigen might be decreased. To further test this hypothesis, we adapted a preferential target recognition assay where we measured TCR-mediated target cell lysis in the absence or presence of BCMA-expressing bystander cells. In the absence of BCMA-expressing bystander cells, TCR^{CMV}:Mock T and TCR^{CMV}:CAR^{BCMA} demonstrated comparable antigen-specific lysis of U266-$HLA^{+/+}$-$BCMA^{-/-}$ pulsed with titrated amounts of pp65-NLV peptide (Figure 5C). In the presence of a 9-fold excess of U266-$HLA^{-/-}$-$BCMA^{+/+}$, TCR^{CMV}:Mock T cells demonstrated unaffected sensitivity toward pp65-NLV peptide. In contrast, TCR^{CMV}:CAR^{BCMA} TRaCR T cells showed markedly reduced sensitivity toward pp65-NLV peptide, requiring an almost 10-fold higher concentration of pp65-NLV peptide to achieve half-maximal killing (EC_{50}) that is comparable to that achieved with TCR^{CMV}:Mock T cells (Figure 5D). TCR^{CMV}:CAR^{BCMA} TRaCR T cells could still reach full clearance of U266-$HLA^{+/+}$-$BCMA^{-/-}$, but required peptide concentrations of 10 ng/mL or more.

In conclusion, CAR engagement reduced the sensitivity of TRaCR T cells toward the cognate peptide HLA (pHLA) antigen. In the presence of the CAR antigen, TRaCR T cells were still capable of responding to TCR antigen, but they required antigen abundance that is magnitudes higher as compared to when stimulated in the absence of the CAR antigen.

TRaCR T cells display higher avidity toward BCMA-expressing target cells

Prompted by our observation that antigen availability may be a contributing factor to preferential target cell recognition, we explored whether cellular avidity may be a driving factor for this phenotype. It is conceivable that TRaCR T cells bind BCMA-expressing target cells with higher cellular avidity as dictated by CAR-BCMA interactions that are of higher affinity and abundance.

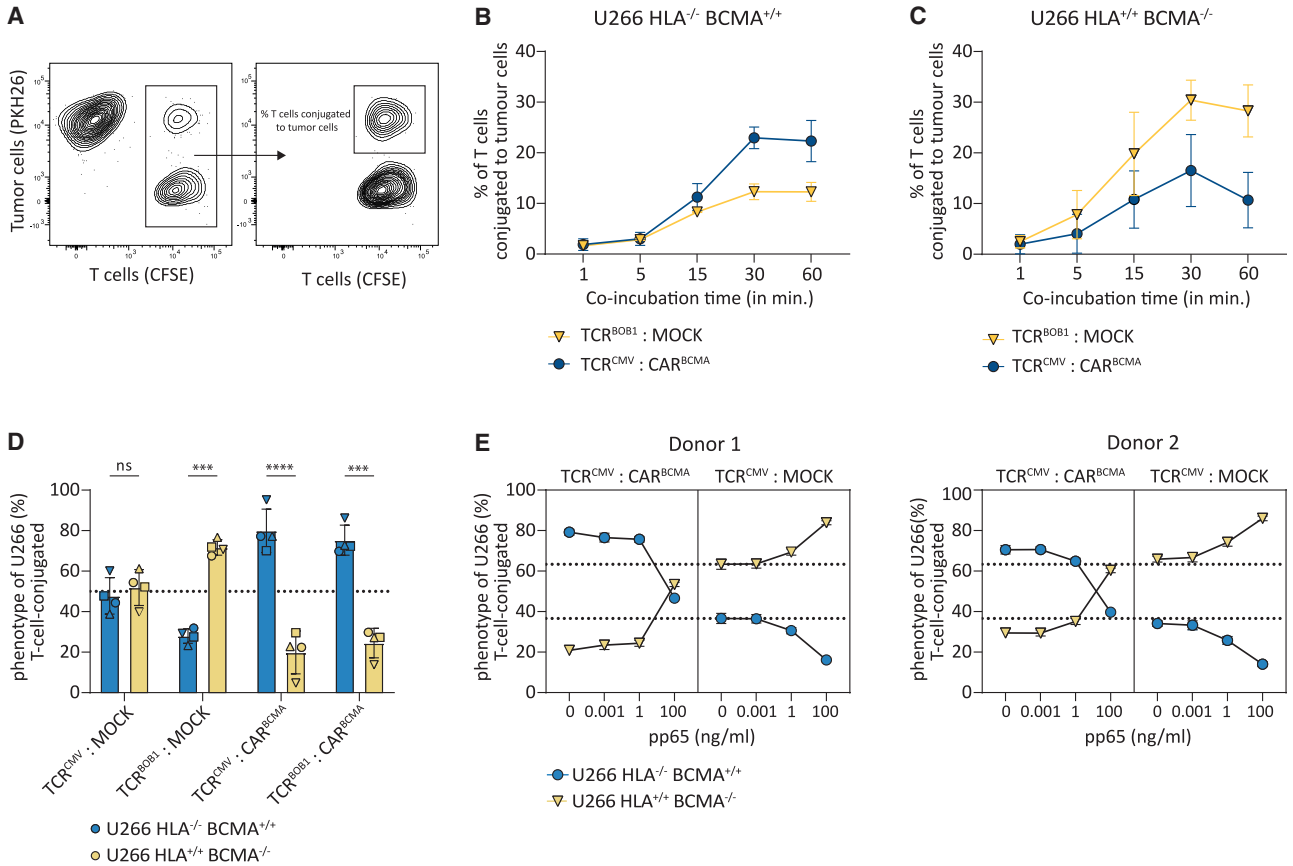

Figure 6. TRaCR T cells display higher cellular avidity toward BCMA-expressing target cells

CFSE labeled T cells were incubated with PKH26 red-labeled tumor cells and allowed to conjugate at 37°C. Cell conjugation was stopped by the addition of PFA, and the percentage of T cells conjugated to tumor cells was assessed using flow cytometry (also see Figure S3). Live cells were identified based on scatterplot, and no duplicate exclusion gate was applied. (A) Representative gating to identify the percentage of T cells conjugated to tumor cells. (B) Kinetics of target cell conjugation specific for CAR-BCMA interaction. (C) Kinetics of target cell conjugation specific for TCR-HLA interaction. (D) Indicated T cell populations were incubated with a 1:1 mix of U266-HLA$^{+/+}$-BCMA$^{-/-}$ and U266-HLA$^{-/-}$-BCMA$^{+/+}$ (final ratio 1 T cell:5 tumor cells). After 30 min of co-incubation, preferential target cell conjugation was assessed by flow cytometry. U266-HLA$^{+/+}$-BCMA$^{-/-}$ and U266-HLA$^{-/-}$-BCMA$^{+/+}$ were discriminated based on the expression of HLA. Data depict averaged triplicate values derived from T cell products generated from four independent donors (indicated by individual symbols). Two-way ANOVA. (E) Preferential target cell conjugation of TCR^{CMV}:CAR^{Mock} or TCR^{CMV}:Mock T cells to U266-HLA$^{+/+}$-BCMA$^{-/-}$ and U266-HLA$^{-/-}$-BCMA$^{+/+}$ in the presence of different amounts of titrated CMV-TCR antigen (pp65-derived NLV peptide). Panels show technical triplicates from different T cell products generated from two independent donors.

We assessed target cell binding kinetics by either antigen-specific TCR-pMHC or CAR-BCMA interactions alone. TCR^{BOB1}:Mock eTCR T cells or TCR^{CMV}:CAR^{BCMA} TRaCR T cells were incubated at 37°C with U266-HLA$^{-/-}$-BCMA$^{+/+}$ or U266-HLA$^{+/+}$-BCMA$^{-/-}$ in an E:T ratio of 1:5 and allowed to conjugate (Figures 6A and S3). Cell conjugation was stopped by the addition of paraformaldehyde (PFA) after set time points ranging from 1 to 60 min. Antigen-specific conjugation mediated by the BCMA-CAR in TCR^{CMV}:CAR^{BCMA} TRaCR T cells was visible from 15 min and after, while plateauing at 30 min, demonstrating about 20% of T cells being bound to U266-HLA$^{-/-}$-BCMA$^{+/+}$ (Figure 6B). TCR^{BOB1}:Mock eTCR T cells showed about 10% non-specific binding to U266-HLA$^{-/-}$-BCMA$^{+/+}$. Conversely, TCR^{BOB1}:Mock eTCR T cells demonstrated an analogous pattern of TCR-pHLA-specific binding of U266-HLA$^{+/+}$-BCMA$^{-/-}$, whereas TCR^{CMV}:CAR^{BCMA} TRaCR T cells also demonstrated at least 10% non-specific binding (Figure 6C). Both TCR- and CAR-mediated binding kinetics appeared similar during this cytometry-based conjugation assay.

We then incubated the single-antigen- or double-antigen-targeting T cell populations with a mix of U266-HLA$^{-/-}$-BCMA$^{+/+}$ and U266-HLA$^{+/+}$-BCMA$^{-/-}$ in a final ratio of 1:2.5:2.5 (T cells:target: target). This allowed us to determine preferential target cell binding within the tumor cells that are bound to T cells (Figure 6D). TCR^{CMV}:Mock T cells showed no significant difference in binding of U266-HLA$^{-/-}$-BCMA$^{+/+}$ or U266-HLA$^{+/+}$-BCMA$^{-/-}$, indicating no preferential target cell binding among non-specific binding. In contrast, TCR^{BOB1}:Mock eTCR T cells preferentially bound tumor cells expressing HLA class I, allowing for pHLA-specific target cell binding. Accordingly, TCR^{CMV}:CAR^{BCMA} TRaCR T cells

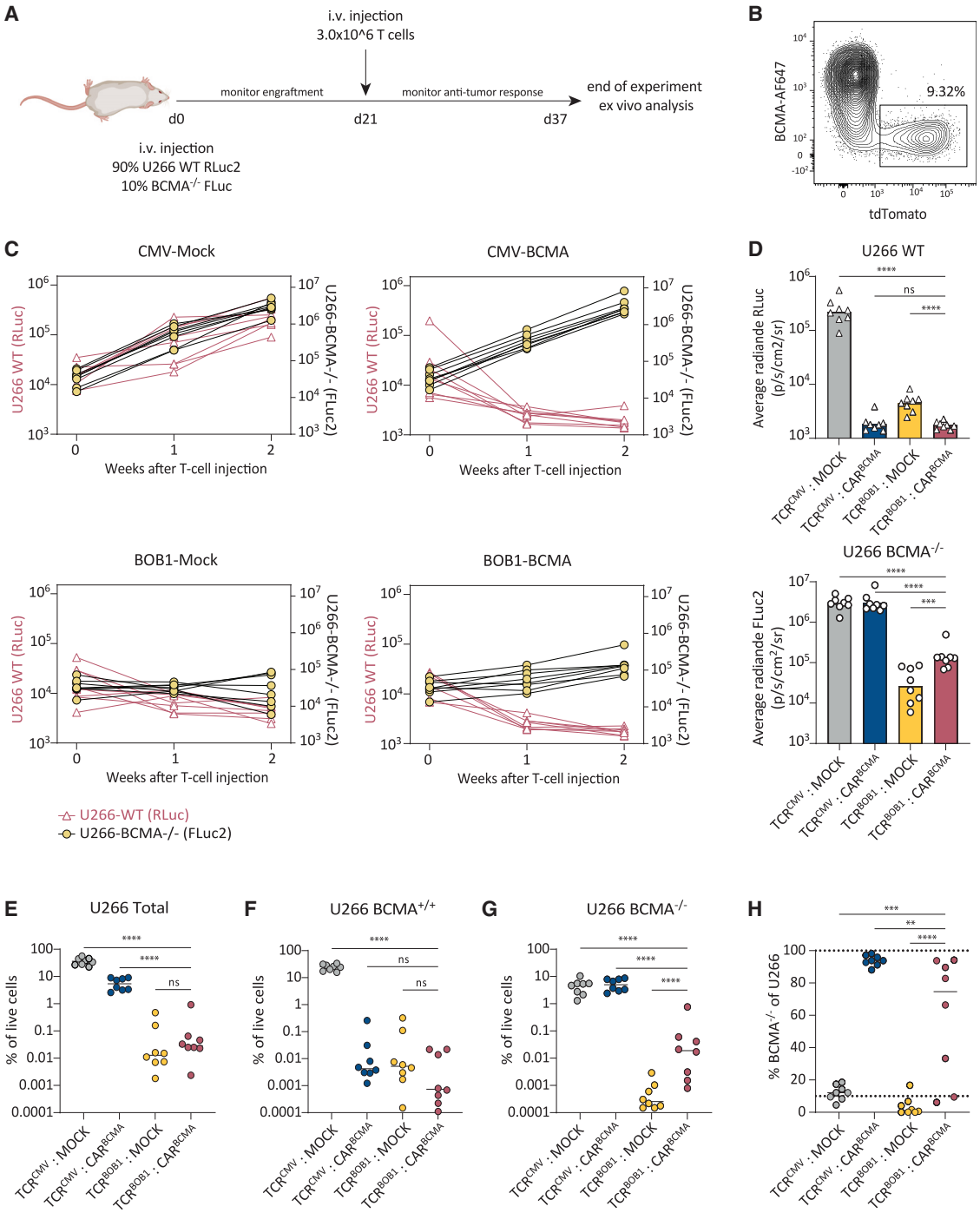

Figure 7. Preferential recognition of BCMA-expressing target cells by TRaCR T cells results in survival advantage of U266-BCMA$^{-/-}$ *in vivo*

(A) Schematic overview of *in vivo* experiment assessing preferential target recognition by TRaCR T cells. NSG mice were inoculated with a 9:1 mix of U266-WT-RLuc and U266-HLA$^{+/+}$-BCMA$^{-/-}$-FLuc2. After an engraftment period of 3 weeks, mice were treated with 3 × 10^6 purified T cell products (n = 8 mice per group). U266-WT-RLuc and U266-HLA$^{+/+}$-BCMA$^{-/-}$-FLuc2 were independently followed using substrate-specific bioluminescence imaging. (B) Frequency of U266-HLA$^{+/+}$-BCMA$^{-/-}$-FLuc2 in tumor cell mix before injection. (C) Bioluminescence of U266-WT-RLuc (left axis, black) and U266-HLA$^{+/+}$-BCMA$^{-/-}$-FLuc2 (right axis, blue) plotted per treatment group. (D) Comparison of bioluminescence of U266-WT-RLuc (above) and U266-HLA$^{+/+}$-BCMA$^{-/-}$-FLuc2 (below) between treatment groups 2 weeks after T cell treatment. (E–G)

(legend continued on next page)

preferentially bound U266-HLA$^{-/-}$-BCMA$^{+/+}$, highlighting target cell conjugation via the CAR-BCMA interaction. Lastly, TCR^{BOB1}:CAR^{BCMA} TRaCR T cells, which principally should be able to conjugate with both target cells antigen specifically, also demonstrated preferential conjugation to target cells expressing BCMA instead of target cells expressing HLA. In accordance with our previous observations, these data demonstrate preferential target cell binding of TRaCR T cells to CAR-antigen-expressing target cells.

Cellular avidity is affected by the affinity of individual interactions as well as by their abundance. We therefore hypothesized that a change in antigen abundance might skew the balance of preferential target cell conjugation. We again exploited the CMV-TCR antigen-abundance titration system using pp65-derived NLV peptide that is naturally absent on U266. We incubated TCR^{CMV}:CAR^{BCMA} TRaCR T cells or TCR^{CMV}:Mock eTCR T cells in a 1:2.5:2.5 mix with U266-HLA$^{-/-}$-BCMA$^{+/+}$ and U266-HLA$^{+/+}$-BCMA$^{-/-}$ that were previously pulsed with increasing concentrations of pp65-NLV peptide. After 30 min of co-incubation of T cells with mixed and peptide-pulsed target cells, we assessed preferential target cell conjugation (Figure 6E). In the absence of pp65-NLV peptide, TCR^{CMV}:CAR^{BCMA} TRaCR T cells preferentially bound U266-HLA$^{-/-}$-BCMA$^{+/+}$. TCR^{CMV}:CAR^{BCMA} TRaCR T cells still preferentially conjugated with U266-HLA$^{-/-}$-BCMA$^{+/+}$ when target cells were pulsed with 0.001 or 1 ng/mL pp65 NLV peptide. However, when pulsed with high concentrations of 100 ng/mL pp65 NLV, the ratio of bound target cells inverted, resulting in more U266-HLA$^{+/+}$-BCMA$^{-/-}$ cells being bound by TCR^{CMV}:CAR^{BCMA} TRaCR T cells than U266-HLA$^{-/-}$-BCMA$^{+/+}$. This observation supports the notion that an increase in antigen abundance can be sufficient to redirect preferential target cell conjugation. Conclusively, preferential target recognition by TRaCR T cells is likely dictated by a disbalance of both the affinity and abundance between TCR-pHLA and CAR-BCMA interactions.

Preferential recognition of BCMA-expressing target cells by TRaCR T cells results in survival advantage of U266-BCMA$^{-/-}$ *in vivo*

We observed that target cell recognition by TRaCR T cells via the TCR was impaired in the presence of CAR-antigen-expressing tumor cells. We hypothesized that this could lead to a selective survival advantage of tumor cells that lost CAR-antigen expression when targeting heterogeneous tumors *in vivo*. To test this hypothesis, we set up an *in vivo* model of heterogeneous myeloma where a fraction of tumor cells were resistant to CAR-mediated targeting but sensitive to TCR-mediated recognition (Figure 7A). Furthermore, we exploited the substrate specificity of different luciferase enzymes to simultaneously follow BCMA-CAR-sensitive and BCMA-CAR-resistant tumor cells through independent bioluminescence imaging (BLI) measurements. To this end, we transduced and sorted U266-HLA$^{+/+}$-BCMA$^{+/+}$ (U266-WT) to express Renilla luciferase (RLuc) specific for coelenterazine and mixed those in a ratio of 9:1 with U266-HLA$^{+/+}$-BCMA$^{-/-}$, which was transduced and sorted to express firefly luciferase (FLuc2) specific for D-luciferin (Figure 7B). This mix of tumor cells was engrafted into NSG mice, and the growth of both tumor cell populations was followed using substrate-specific BLI.

Three weeks after engraftment, mice inoculated with this heterogeneous tumor cell population were treated with 3×10^6 TCR^{CMV}:Mock, TCR^{CMV}:CAR^{BCMA}, TCR^{BOB1}:Mock, or TCR^{BOB1}:CAR^{BCMA} T cells. T cells used for this experiment were generated by re-stimulation and sequential transduction of CMV-TCR or BOB1-TCR transduced T cells as described earlier to exclude confounding differences in receptor expression. In mice treated with TCR^{CMV}:Mock T cells, tumor growth of both BCMA-expressing U266-WT-RLuc and CAR-refractory U266-BCMA$^{-/-}$FLuc2 progressed (Figure 7C). Mice receiving TCR^{CMV}:CAR^{BCMA} T cell treatment did not show any reduction of U266-BCMA$^{-/-}$FLuc2 compared to control T cells, while U266-WT-RLuc was clearly reduced. Mice treated with TCR^{BOB1}:Mock T cells displayed reduction in BLI from both U266-WT-RLuc and U266-BCMA$^{-/-}$FLuc2. Mice treated with TCR^{BOB1}:CAR^{BCMA} TRaCR T cells demonstrated a decrease in BLI U266-WT-RLuc, whereas the BLI of U266-BCMA$^{-/-}$FLuc2 increased further.

When comparing BLI 2 weeks after treatment, reduction in BLI of U266-WT-RLuc was comparable between TCR^{BOB1}:CAR^{BCMA} and TCR^{CMV}:CAR^{BCMA} TRaCR T cells and superior to that of mice treated with TCR^{BOB1}:Mock eTCR T cells (Figure 6C). TCR^{BOB1}:CAR^{BCMA} TRaCR T cells could decrease the burden of U266-BCMA$^{-/-}$FLuc2, however, to a significantly lower extent as compared to TCR^{BOB1}:Mock eTCR T cells (Figure 7D). TCR^{CMV}:CAR^{BCMA} T cells were unable to induce any anti-tumor activity against U266-BCMA$^{-/-}$FLuc2 as compared to TCR^{CMV}:Mock T cell-treated mice (Figure 7D). This discrepancy highlights that TCR^{BOB1}:CAR^{BCMA} TRaCR T cells indeed exerted preferential activity versus targets expressing BCMA *in vivo*.

On day 16 after T cell treatment, mice were sacrificed and assessed for the presence and phenotype of tumor cells in the bone marrow. Both TCR^{BOB1}:Mock and TCR^{BOB1}:CAR^{BCMA} T cells efficiently and comparably reduced the overall tumor burden compared to TCR^{CMV}:Mock and TCR^{CMV}:CAR^{BCMA} T cells (Figure 6E). Clearance of tumor cells that express BCMA was comparable between TCR^{CMV}:CAR^{BCMA}, TCR^{BOB1}:Mock, and TCR^{BOB1}:CAR^{BCMA} T cells (Figure 7F). U266-BCMA$^{-/-}$FLuc2 was not cleared by TCR^{CMV}:CAR^{BCMA} T cells, whereas TCR^{BOB1}:CAR^{BCMA} TRaCR T cells could significantly reduce the burden of tumor cells lacking

Frequency of indicated tumor cell populations in the bone marrows of sacrificed mice on day 16 after T cell treatment as measured by flow cytometry. (D–G) Ordinary one-way ANOVA with Dunnett's multiple comparisons post hoc test on log-transformed data comparing TCR^{BOB1}:CAR^{BCMA} TRaCR T cells to other groups. (H) Frequency of tumor cells of an BCMA$^{-/-}$-tdTomato^{+} phenotype of all U266 cells. Dotted line indicates 10% as the reference level for pre-injection frequency. Ordinary one-way ANOVA with Dunnett's multiple comparisons post hoc test comparing TCR^{BOB1}:CAR^{BCMA} TRaCR T cells to other groups.

BCMA expression. However, mice treated with TCR^{BOB1}:CAR^{BCMA} TRaCR T cells still displayed on average 100-fold higher frequencies of tumor cells lacking BCMA expression as compared to mice treated with TCR^{BOB1}:Mock eTCR T cells (Figure 7G). This survival advantage of U266-$BCMA^{-/-}$FLuc2 is further reflected in the change in the relative proportion of tumor cells lacking BCMA within the remaining tumor cells (Figure 7H).

These results demonstrate that CAR-mediated preferential target recognition limited the dual-antigen specificity of TCR and CAR dual-receptor-edited T cells in an *in vivo* model of heterogeneous myeloma. TCR^{BOB1}:CAR^{BCMA} TRaCR T cells preferentially targeted tumor cells expressing BCMA. This resulted in a selective survival advantage of tumor cells lacking expression of the CAR antigen, highlighting potential translational implications for TCR- and CAR-based multi-antigen-targeting strategies.

DISCUSSION

We explored the prospect of engineering T cells to express a transgenic TCR alongside a CAR to install dual-antigen specificity on T cells. We demonstrated that such T cells, called TRaCR T cells, can be generated, either from a single expression vector or through sequential transductions. T cells transduced with a single expression vector demonstrated surface expression of both the CAR and the transgenic TCR, although at lower expression levels as compared to T cells transduced to express either the transgenic TCR or the CAR alone, probably as a consequence of the increased size of the viral expression vector. We showed that T cells generated in this way displayed general dual specificity. The presence of either the CAR antigen or the respective TCR antigen was sufficient to induce TRaCR T cell activation, resulting in cytokine production and T cell proliferation, as well as in target cell killing. These observations indicate that TRaCR T cells could potentially serve as an attractive alternative to T cells engineered expressing a CAR or a transgenic TCR alone. However, we also observed that activation via the CAR conferred preferential target recognition, resulting in diminished sensitivity of TCR-mediated target cell recognition in the presence of BCMA-expressing target cells. This was independent of the manufacturing protocol and extendable to other TCRs. Consequentially, in a murine xenograft *in vivo* model of heterogeneous MM where 10% of the tumor cells lacked BCMA expression, clearance of those tumor cells was inferior as compared to mice treated with BOB1 TCR single-antigen-targeting T cells.

CAR-mediated signaling has been described to differ in several aspects from TCR-mediated signaling, allowing for some explanation on how CAR-engagement may render TRaCR T cells hyporesponsive toward stimulatory pMHC. Generally, CARs bind antigen with much higher affinity than do TCRs. As antigen recognition of CARs is determined by an antibody-derived scFv, CARs usually exhibit binding affinities in the nanomolar range. The affinity of the 11C5.3 antibody (that served as the basis for the scFv of the BCMA CAR used presently) has also been described to fall into this range with 3.3 nM.[36] However, TCRs usually have a micromolar affinity for their antigen. While we do not have biophysical data available for the BOB1 TCR used in this study, we can assume that the affinity is likely magnitudes lower, taking into account that this TCR has not undergone affinity maturation. Similarly, we assume that the affinity of the CMV TCR falls into the micromolar range as is typically observed for virus-specific TCRs. The NY-ESO-1 TCR used in this study, a clinically validated, affinity-matured TCR derived from the HLA-autologous repertoire, has a reported affinity of 730 nM.[35,37] For all three TCR-BCMA-CAR combinations, we observed preferential target recognition through the BCMA CAR. It is conceivable that TRaCR T cells bind target cells through the CAR molecules with higher affinity. Moreover, the actual amount of stimulatory antigen may differ greatly for pHLA or a full-length surface protein such as BCMA and could consequently affect differential receptor engagement.[38,39] Together, the differences in affinity and antigen abundance may result in differential cellular avidity toward target cells. This notion is supported by the data from the peptide titration experiments. We revealed that TRaCR T cells were able to still recognize targets via TCR-mediated recognition in the presence of CAR antigen. However, TCR-mediated target recognition required much higher, supraphysiological concentrations of agonistic peptide to achieve similar target cell clearance in the presence of CAR-antigen-expressing bystander cells than in the absence of CAR antigen. Analogously, TRaCR T cells conjugated preferentially with BCMA-expressing target cells. Only after pulsing of high amounts of stimulatory peptide could the cellular avidity of TRaCR T cells be skewed toward HLA-expressing, BCMA-negative target cells.

Moreover, CARs are described as transducing signals into T cells more rapidly than do TCRs, while forming non-classical immune synapses independently of the endogenous TCR.[40,41] Together, these effects may lead to the sequestration of downstream signaling molecules toward CAR molecules that subsequently become unavailable for productive signaling through the TCRs, resulting in hyporesponsive TCR-pMHC interactions.

Conceptually, it could be interesting to assess the degree of preferential target recognition of TRaCR T cells, where both the introduced TCR and the CAR fall into the same affinity range and in the presence of different amounts of stimulatory antigens. It must be noted, however, that this would imply either substantial reduction of the affinity of the CAR or, conversely, enhancement of the affinity of the respective TCR. Both approaches might present downsides for clinical translation: lower CAR affinity can result in diminished antigen sensitivity, while unnatural affinity enhancement of TCRs can install unwanted off-target recognition patterns.[42–44]

MM is described as a malignancy with substantial intra-tumoral and spatial heterogeneity.[45] The rationale of dual targeting is based on the assumption that some MM cells will display reduced expression or complete absence of BCMA, making them unamenable to BCMA-CAR-mediated recognition. Especially for those cells, it is crucial that they be targeted through recognition of a different antigen. Critically, our results suggest that in the presence of BCMA-expressing

target cells, target cell recognition through the transgenic TCR expressed on TRaCR cells was impaired. We therefore advise caution when using TRaCR T cells as a strategy to circumvent antigen escape after cellular therapy of MM. Instead, it might be more advisable to manufacture a mix of T cells separately engineered to express either a CAR or a transgenic TCR at a time. Furthermore, given the HLA restriction of TCRs, it would be necessary to design multiple CAR-TCR combinations depending on patient characteristics. Keeping in mind an HLA-diverse patient population, it could actually be more economical to combine separate products rather than design multiple TRaCR combinations.

Analogous to our findings, dual-specific CAR T cells have been observed to display issues that could be related to preferential target recognition. In a clinical trial using CD19/CD22 bispecific CAR T cells, antigen escape was driven mainly by the loss of CD19 expression, whereas CD22 expression was maintained.[46] In a preclinical study using BCMA/CS1 bispecific CAR T cells for the treatment of heterogeneous MM, relapsing MM cells were mostly of a $BCMA^{-}/CS1^{+}$ phenotype.[47] While many dual-specific CAR T cell therapies are being developed preclinically and clinically, our observations highlight that dual-antigen specificity should be critically evaluated in settings of heterogeneous antigen exposure.[48–51] Dual-antigen-targeting strategies combining TCR-mediated target recognition with CAR-based antigen recognition have already been suggested; however, preferential antigen recognition remained limitedly explored in those studies.[52–54]

Our results have potential implications for other multi-antigen-targeting strategies incorporating TCR-CAR combinations. For example, it has been suggested to engineer tumor-infiltrating lymphocytes to express CARs (CAR-TILs) to install dual-targeting specificity.[55,56] It is possible that CAR-TILs similarly would be limited in their dual-targeting specificity through their native TCR when activated through the CAR. While our study is only limitedly extendable to settings of endogenously expressed TCRs as we solely assessed activation through retrovirally expressed TCRs, another report presented similar observations using CAR-engineered alloreactive murine T cells.[57] In that study, alloreactive murine T cells displayed a diminished potential to induce graft-versus-host disease when targeting leukemia cells *in vivo*, indicating that preferential target recognition of CAR T cells may extend to endogenously expressed TCRs.

In conclusion, we describe the development of dual-antigen-specific T cells through combined TCR and CAR engineering. Our data highlight limitations associated with installing dual specificity into single T cells and warrant careful consideration when following this strategy for multi-antigen-targeting T cell therapy of MM.

MATERIALS AND METHODS

Cell culture

U266 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), glutamine, and penicillin/streptomycin. T cells were cultured in T cell medium (TCM) consisting of IMDM supplemented with 5% FCS, 5% human serum, 10% FCS, glutamine, penicillin/streptomycin, and 100 IU/mL interleukin-2. T cells were maintained at a culturing density not exceeding 1×10^6 cells/mL. Generation of U266-$B2M^{-/-}$ and U266-$BCMA^{-/-}$ through CRISPR-Cas9 engineering was described earlier.[24]

Generation of T cell products

$CD8^+$ T cells were derived from frozen healthy donor PBMCs using direct CD8 MACS (Miltenyi). Isolated $CD8^+$ T cells were activated with 0.8 μg/μL phytohemagglutinin and irradiated autologous feeder cells and cultured in TCM. At 48 h after activation, T cells were transduced with retroviral supernatants adhered to retronectin-coated (Takara) non-tissue culture-treated plates. On day 7 after activation, T cells were MACS purified for transgene expression (either dNGFR or murine TCR-β) and quality checked by flow cytometry. Purified TCR- and CAR-transduced cells were used for experiments between days 9 and 14 after initial activation. For TRaCR T cells generated by sequential stimulation and transduction, purified mTCR expressing eTCR T cells were re-stimulated using the protocol outlined above on day 9 after initial stimulation. For the second transduction, T cells were transduced 24 h after re-stimulation and enriched for dNGFR expression on day 6 after restimulation.

IFN-γ ELISA

Supernatant of cocultures was analyzed for IFN-γ release by T cells using enzyme-linked immunosorbent assay (ELISA) (Diaclone). The protocol was adjusted for 384-well plates. Supernatants were diluted 5× and 125×. If values of the 5× dilution exceeded the linear range of the standard curve, then values obtained from the 125× dilution were used instead. Data display combined values derived from either 5× or 125× dilutions using appropriate back-calculations of concentrations.

Flow cytometry

Unless specified otherwise, cells were washed and stained in 96-well U-bottom plates. Sytox blue (Life Science Technologies) was used as a viability dye in a 1:1,000 dilution. Data were acquired on a Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 software. Experiments involving cell counts were performed using isovolumetric measurements. CAR expression was assessed using goat-anti-mouse (GaM) secondary antibodies (Jackson ImmunoResearch). GaM-stained samples were washed twice and blocked with 5% mouse serum at 15 min at room temperature before undergoing counterstaining to minimize cross-reactivity of the GaM antibody with other staining antibodies. In later experiments, CARs were stained using an antibody specific for the Whitlow/218 linker that is part of the scFv (Cell Signaling Technology).[58]

Preferential killing assays

In a 96-well round-bottom plate, CD8 $TCR^{BOB1/NY\text{-}ESO\text{-}1/CMV}$:Mock or $TCR^{BOB1/NY\text{-}ESO\text{-}1/CMV}$:$CAR^{BCMA}$ (E) T cells were cocultured with U266-$BCMA^{-/-}$ (T_1) at a 1:1 E:T ratio, U266 $B2M^{-/-}$ (T_2) at a 1:1 E:T ratio, and a mix of both U266 targets at a 1:1:9 or 1:9:1

E:T_1:T_2 ratio. After 24 h, survival of the tumor cells was assessed by flow cytometry. Sytox blue (Thermo Fisher) was used to determine cell viability, and the following antibodies were taken along for identification of the tumor and T cells: anti-CD2 BV786-conjugated (BD Biosciences), anti-CD8 AF-700-conjugated (Invitrogen), anti-HLA-A*02 fluorescein isothiocyanate-conjugated (BD Biosciences), and anti-BCMA AF647-conjugated (BioLegend). U266 were tdTomato positive. Target cells of interest, either U266 BCMA$^{-/-}$ or U266 B2M$^{-/-}$, were discriminated based on HLA and BCMA expression. Gating is shown in Figure S2. Data were acquired on a Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version10 software.

Luminescence-based killing assay

For the preferential killing assay combined with an NLV-peptide titration, TCR^{CMV}:Mock or TCR^{CMV}:CAR^{BCMA} were cocultured with luciferase-positive U266-BCMA$^{-/-}$, and luciferase-negative U266-B2M$^{-/-}$ cells at a 1:1:0 or a 1:1:9 ratio. The U266-BCMA$^{-/-}$ cells were pulsed with differing concentrations of CMV-pp65-derived NLV peptide ranging from 0 to 1,000 ng/mL. Culture media contained 1 mM D-luciferin. After 24 h, the survival of luciferase-positive U266-BCMA$^{-/-}$ cells was determined by measuring bioluminescence on a SpectraMax i3x Microplate Reader (Molecular Devices). Measurements were performed at 37°C.

Cell conjugation assay

For cell conjugation experiments, we followed a modified protocol derived from Burshtyn and Davidson.[59] Prior to experiments, T cells were labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE), while target cells were labeled with PKH26 Red according to the manufacturer's instructions (Sigma-Aldrich) and subsequently stained with anti-HLA-A*02 BV480-conjugated (BD Biosciences). A total of 20,000 T cells were mixed with 100,000 target cells in 150 μL total volume in 5 mL fluorescence-activated cell sorting (FACS) tubes on ice and then incubated in a water bath at 37°C for the indicated times (1–60 min). At the end of the incubation, 300 μL of 0.5% PFA solution was added (0.33% final PFA concentration), immediately followed by brief vortexing at high speed for exactly 3 s and placed on ice before acquisition by flow cytometry. No additional washing steps were performed. Live cells were identified based on scatterplot, and no duplet exclusion gate was applied. T cells conjugated to tumor cells were identified by CFSE/PKH26 red double-positive cells. The distinction between U266-BCMA$^{-/-}$ and U266-B2M$^{-/-}$ was made based on the expression of HLA-A*02. T cell products were derived from HLA-A*02-negative donors. Data were acquired on a Fortessa X-20 flow cytometer (BD Biosciences) and analyzed using FlowJo version 10 software.

NSG xenograft models

In-house bred NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice (NOD *scid* gamma [NSG]) (The Jackson Laboratory) were used for the xenograft experiments. U266-WT cells were transduced to express RLuc and sorted based on dNGFR marker gene expression. U266-BCMA$^{-/-}$ were transduced to express FLuc2 and sorted based on tdTomato marker gene expression. NSG mice were inoculated intravenously by tail vein injection with a mix of 1.8×10^6 U266-WT-RLuc and 0.2×10^6 U266-BCMA$^{-/-}$-FLuc2 in 100 μL PBS per mouse. BLI measurements were performed using a LAGO imaging system (Spectral Instruments). For RLuc luminescence, freeze-dried coelenterazine was reconstituted immediately before use and injected retro-orbitally into anesthetized mice at 100 μg/100μL. Imaging was performed within 1 min after retro-orbital injection of coelenterazine. For imaging of FLuc luminescence, mice were injected subcutaneously with 150 μL 7.5 mM D-luciferin. This study was approved by the Dutch National Ethical Committee for Animal Research (AVD116002017891) and was performed in accordance with Dutch laws for animal experiments. At the end of the experiments, mice were euthanized using CO_2, and femurs were extracted for downstream analysis.

Ex vivo analysis

Femurs were removed from euthanized mice, and nucleated bone marrow cells were extracted from femurs using spin-isolation.[60] Cells isolated from left and right femurs of individual mice were pooled. Erythrocytes were lysed using ammonium chloride-based red blood cell lysis buffer. Cell suspensions were strained through a 30-μm filter and stained for downstream FACS analysis. A minimum of 2×10^5 live events were acquired per sample.

DATA AVAILABILITY

Data supporting the findings of this study are available from the corresponding authors upon reasonable request.

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AUTHOR CONTRIBUTIONS

T.L.A.W.: conceptualization, data curation, formal analysis, investigation, methodology, supervision, visualization, writing – original draft, and writing – review & editing. T.P.: data curation, formal analysis, investigation, methodology, validation, and writing – review & editing. M.H.M.: data curation, formal analysis, investigation, and visualization. D.F.G.R.: investigation and methodology. M.F.T.: investigation. A.K.W.: investigation. R.S.H.: investigation and methodology. J.H.F.F.: supervision and writing – review & editing. M.H.M.H.: conceptualization, funding acquisition, project administration, resources, supervision, and writing – review & editing.

DECLARATION OF INTERESTS

M.H.M.H. and J.H.F.F. hold patents related to the 4G11 BOB1 TCR used in this study (EP3215522). The 4G11 BOB1 TCR is licensed to Miltenyi Biotech. Miltenyi Biotech provided research funding to M.H.M.H.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2025.02.035.

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