CANCER

TCR beta chain-directed bispecific antibodies for the treatment of T-cell cancers

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Immunotherapies such as chimeric antigen receptor (CAR) T cells and bispecific antibodies redirect healthy T cells to kill cancer cells expressing the target antigen. The pan-B cell antigen targeting immunotherapies have been remarkably successful in treating B cell malignancies. Such therapies also result in the near complete loss of healthy B cells, but this depletion is well-tolerated by patients. Although analogous targeting of pan-T cell markers could in theory help control T-cell cancers, the concomitant healthy T cell depletion would result in severe and unacceptable immunosuppression. Thus, therapies directed against T-cell cancers require more selective targeting. Here we describe an approach to target T-cell cancers through T-cell receptor (TCR) antigens. Each T cell, normal or malignant, expresses a unique TCR β chain generated from one of thirty TCR β chain variable gene families (TRBV1 to TRBV30). We hypothesized that bispecific antibodies targeting a single TRBV family member expressed in malignant T cells could promote killing of these cancer cells, while preserving healthy T cells that express any of the other 29 possible TRBV family members. We addressed this hypothesis by demonstrating that bispecific antibodies targeting TRBV5-5 (α -V5) or TRBV12 (α -V12) specifically lyse relevant malignant T cell lines and patient-derived T-cell leukemias *in vitro*. Treatment with these antibodies also resulted in major tumor regressions in mouse models of human T-cell cancers. This approach provides an off-the-shelf, T-cell cancer selective targeting approach that preserves enough healthy T cells to maintain cellular immunity.

INTRODUCTION

T-cell cancers are a heterogeneous group of malignancies that comprises about 15% of non-Hodgkin's lymphomas (1) and 20% of acute lymphoblastic leukemias (ALL) (2, 3). Outcomes of T-cell lymphomas and relapsed T-cell ALL (T-ALL) are worse than those for equivalent B cell malignancies, with an estimated 5-year survival of only 32% in T-cell lymphomas (4) and 7% in relapsed T-ALL (5). There are several targeted immunotherapeutic agents for B cell malignancies, including monoclonal antibodies (6), bispecific T cell engaging antibodies (BsAb) (7–10) and chimeric antigen receptor (CAR) T cells (11–14). But there are few targeted therapeutic agents for T-cell malignancies. Malignant B or T cells do not express cell-

surface antigens that are distinct from their non-cancerous counterparts. As a result, B cell immunotherapies target pan-B cell antigens such as CD19 or CD20, which is feasible because the associated normal B cell aplasia is clinically well-tolerated. A similar strategy targeting pan-T cell antigens is unfeasible because the resultant T cell depletion would lead to a clinically unacceptable and profound immunosuppression.

The $\alpha\beta$ T-cell receptor (TCR) is a trans-membrane heterodimer that is expressed on normal T cells and mature T-cell cancers including peripheral T-cell lymphomas (PTCL), angioimmunoblastic T-cell lymphomas (AITL) (15, 16), T-cell prolymphocytic leukemia (T-PLL), adult T-cell

leukemia/lymphoma (ATLL) (17), cutaneous T-cell lymphomas (CTCL) (18) and a substantial fraction (30-50%) of T-ALL (19, 20). The TCR β chain (TCRB) germline locus on chromosome 7 is composed of 68 variable (V) gene segments, along with 2 diversity (D), 13 joining (J), and 2 constant (C) gene segments (21). The T-cell receptor β chain variable gene (TRBV) segments are classified into 30 TRBV families based on nucleotide sequence similarity (22). During T cell development, the V, D, and J TCRB germline gene segments undergo rearrangements leading to a continuous V-D-J transcript which codes for a unique TCRB chain in each T cell (23). This VDJ recombination results in expression of one of the 30 TRBV gene families on the surface of each T cell. Each TRBV is expressed on the surface of 1 to 5% of the total normal human peripheral blood T cells (24). In contrast, clonal T-cell cancers express only one TRBV, providing a potential opportunity to selectively deplete clonal T-cell cancers while retaining the majority of the normal T cells. Murine studies also confirmed that deletion of T cells expressing individual TRBVs have normal immune responses (25). Clonal T-cell cancers also express only one of the two TCR β chain constant regions (TRBC1 or TRBC2), whereas normal T cells express a mixture of both TRBC1 and TRBC2. Taking advantage of this cancer-specific expression, a study with TRBC1 targeting CAR T cells demonstrated efficacy in experimental models of TRBC1⁺ malignancies while preserving all TRBC2⁺ functional T cells (16). However the high cost and patient-specific cellular engineering involved with CAR T cells currently limits widespread adoption of CAR T cell therapeutics (26, 27). BsAbs are a family of molecules engineered to bind two disparate antigens. BsAbs are constructed by linking two distinct single-chain variable fragments (scFv) (28, 29). Usually one scFv is directed against a cancer tissue specific antigen and the other scFv targets the T cell activating molecule CD3. The dual binding of cancer cell and T cell results in T cell mediated cancer cell death. BsAbs have demonstrated activity in a range of B cell lymphomas and leukemias (8, 9) and provide a viable, off-the-shelf alternative targeted therapeutic option to CAR T cells. Several cancer-directed bispecific antibodies are currently undergoing clinical trials (28), but none involve treating T-cell cancers.

Here we describe the generation and testing of a TRBC and two TRBV targeting BsAbs for the treatment of T-cell cancers. We demonstrate that the TRBC-targeting BsAb eradicates both the T-cell cancers and the vast majority of healthy human T cells due to bidirectional T cell killing. However, TRBV-targeting BsAbs deplete cancerous T cells in vitro and in vivo while preserving the majority of normal T cells, thereby avoiding treatment-related immunosuppression and providing a potential therapeutic option for treating T-cell malignancies.

RESULTS

BsAb primarily target normal T cells expressing the TCRB antigen of interest

T-ALL (30), CTCL (31, 32), AITL (33), PTCL, ATLL (34) and T cell large granular lymphocyte leukemia (T-LGL) (35) have all been reported to demonstrate clonal expression of TRBV5 or TRBV12 families (Fig. 1A). Using anti-TRBV5-5 scFv (K_d 25.2 nM) and anti-TRBV12 scFv (K_d 2.6 nM) sequences, we generated anti-TRBV5-5 and anti-TRBV12 BsAbs (henceforth denoted “ α -V5” and “ α -V12”) for selective targeting of TRBV5-5⁺, or TRBV12⁺ T cells, respectively (Fig. 1B, fig. S1A, and table S1). Similarly, using anti-TRBC1 scFv (K_d 0.4 nM) sequence, we generated anti-TRBC1 BsAbs (henceforth denoted “ α -C1”) for selective targeting of TRBC1⁺ T cells (Fig. 1B and table S1). Analytic chromatography showed monomeric BsAbs with >99% purity (fig. S1B, C). Thermal stability of α -V12 and α -V5 were evaluated using differential scanning fluorimetry. α -V12 showed two melting temperatures (T_m) at 59°C and 77°C, and α -V5 presented a single T_m at 78°C (fig. S1D, E). These data suggest that for α -V12, the anti-TRBV12 scFv unfolds at 59°C and the anti-CD3 scFv unfolds at 77°C, whereas for α -V5, both the anti-TRBV5-5 scFv and the anti-CD3 scFv unfold at 78°C. We found that approximately 1.5 to 2% and 3.5% to 5% of normal human T cells isolated from 5 healthy donors express TRBV5-5 and TRBV12 (Fig. 1C, D), respectively, consistent with past reports (24). Approximately 35-45% human T cells isolated from the same donors expressed TRBC1 and the rest expressed TRBC2 (Fig. 1C, E) (16). As expected, in vitro exposure of T cells from healthy individuals to α -V5 and α -V12 treatment resulted in complete loss of the TRBV5-5⁺ and TRBV12⁺ cells, respectively (Fig. 1C, D and fig. S2A). Similarly, exposure of T cells from healthy individuals to α -C1 resulted in a substantial loss of TRBC1⁺ T cells (Fig. 1C, E and fig. S2B). However, there was a major difference in the loss of the non-targeted T cells mediated by the TRBV- and TRBC-specific BsAbs. On average, the α -V5 BsAb depleted 14.1% of the T cells not expressing TRBV5-5, and α -V12 eradicated 13.3% of the T cells not expressing TRBV12 (Fig. 1C). In contrast, α -C1 eradicated, on average, 80.0% of the T cells not expressing TRBC1 (Fig. 1C). Consequently, α -C1 resulted in depletion of the majority of the total T cells, while α -V5 or α -V12 preserved the majority of T cells (Fig. 1C). To confirm that BsAb mediated TCR internalization or TCR epitope blocking are not interfering with subsequent antibody based analysis of different T cell subtypes, we labeled TRBV5⁺, TRBV12⁺ or TRBC1⁺ target T cells with CellTrace Violet. As expected, α -V5 and α -V12 exposure led to depletion of CellTrace Violet-labeled TRBV5⁺ and TRBV12⁺ cells (fig. S2C, D), and α -C1 caused substantial loss of both TRBC1⁺ and TRBC2⁺ cells (fig. S2E, F).

Bidirectional killing accounts for BsAb targeting of T cells that do not express the relevant TRBV or TRBC

We next asked why these BsAbs resulted in killing of T cells not expressing the relevant TRBV or TRBC chain. One hypothesis was that the BsAbs were not entirely specific for the targeted TRBV or TRBC chains, causing off-target cell killing. To test this hypothesis, we depleted TRBC1⁺ cells from human T cells, then exposed them to α -C1. After depletion of T cells expressing TRBC1, exposure to α -C1 did not result in statistically significant killing of the remaining T cells ($P = 0.09$, fig. S2G). Similarly, after depletion of T cells expressing either TRBV5 or TRBV12, exposure of α -V5 or α -V12 did not result in statistically significant killing of the remaining T cells ($P = 0.10$ and $P = 0.27$ respectively, fig. S2H). Additionally, a pure population of TRBV5⁺ or TRBV12⁺ T cells experienced almost complete cell loss after exposure to α -V5 and α -V12 respectively (fig. S2I). These results excluded the hypothesis that the BsAbs killed T cells not expressing the relevant TRBV or TRBC chain because of promiscuous off-target activity toward other TRBV or TRBC chains. The effects of all three BsAbs were exquisitely dependent on the presence of the relevant TRBV or TRBC chains in the treated T cells.

The BsAb molecules (Fig. 1B) are composed of one scFv arm interacting with a TRBC or a TRBV region expressed only by the target T cell subset, and the other scFv arm interacting with CD3 ϵ subunit expressed on all T cells (36). An alternate hypothesis to explain the killing of T cells not expressing relevant TRBC or TRBV chains by α -C1, α -V5 and α -V12 involves bidirectional killing, where crosslinking by the BsAbs induce activation of both “effector” and “target” T cells, thereby killing the crosslinked “effector” T cells (Fig. 2A). We thus suspected that the α -C1 crosslinking could activate TRBC1⁺ T cells and kill the conjugated TRBC2⁺ “effector” T cells. This would result in the killing of both TRBC1- and TRBC2-expressing T cells, leading to the observed near complete T cell depletion (Fig. 1C). Similarly, α -V5 or α -V12 would result in bidirectional killing of T cells not expressing TRBV5-5 or V12. In contrast, BsAbs used in non-T-cell cancer targeting strategies are directed against cancer-cell surface antigens, resulting in unidirectional T cell activation and killing. To test this hypothesis, we generated three additional BsAbs. These used the identical TRBV or TRBC scFvs described above but the α -CD3 scFv was substituted with an α -CD19 scFv (Fig. 2B and table S1). The α -CD19 scFv has been commonly used for BsAb mediated-targeting of B cells (10, 37). This allowed us to test whether TRBC1, TRBV5-5 or TRBV12 engagement is sufficient for T cell activation and subsequent killing of CD19⁺ NALM6 B cells. As a positive control, we used a conventional BsAb targeting CD19, in which an scFv against the CD3 is joined to the CD19-specific scFv. We utilized CD19 knock out NALM6 B cells as negative control target cells (fig. S3A). In the presence of any of the four BsAbs, co-culture of

CD19⁺ target NALM6 B cells with normal T cells resulted in cytokine production including interferon gamma (IFN γ), interleukin 2 (IL-2), tumor necrosis factor alpha (TNF α) and IL-10 (Fig. 2C and fig. S3B). Furthermore, α -CD3-CD19 or α -C1-CD19 BsAbs resulted in the expression of T cell activation markers including CD25, inducible T cell co-stimulatory (ICOS), 4-1BB, and of exhaustion markers such as lymphocyte-activation gene 3 (LAG-3) and programmed death 1 (PD-1) (fig. S3C). These two BsAbs also promoted target NALM6 B cell killing (Fig. 2D). Co-culture of T cells and NALM6 B cells with α -V5-CD19 or α -V12-CD19 also promoted a modest increase in LAG-3 expression and tumor cell killing. The T cell cytokine production and NALM6 B cell cytotoxicity was dependent on NALM6 CD19 expression, as deletion of CD19 on NALM6 cells abrogated these effects (Fig. 2C, fig. S3B and Fig. 2D). We did not observe any difference in IFN γ production or NALM6 B cell cytotoxicity between wild type NALM6 B cells and NALM6 B cells expressing reduced CD19 with our BsAbs ($P = 0.61$, $P = 0.58$, $P = 0.43$, $P = 0.68$, and $P = 0.29$, $P = 0.38$, $P = 0.70$, $P = 0.29$, fig. S3A, D and E). To characterize the specificity of these effects, we depleted TRBV5⁺ and TRBV12⁺ T cells from the T cell pool prior to co-culturing them with NALM6 B cells in the presence of the BsAbs. The remaining T cells remained inactivated, as shown by their inability to generate IFN γ after co-culture with NALM6 B cells in the presence of α -V5-CD19 and α -V12-CD19 (Fig. 2E). Similarly, depleting TRBV5⁺ and TRBV12⁺ T cells left the remaining T cells unable to kill NALM6 B cells (Fig. 2F). Exposure to α -CD3-CD19 and α -C1-CD19 resulted in considerably higher IFN γ production and NALM6 cell cytotoxicity than exposure to α -V5-CD19 and α -V12-CD19 (Fig. 2E, F). This is likely because 35-45% human T cells express TRBC1 while 1.5% to 5% of normal T cells express TRBV5-5 or TRBV12 (Fig. 1C, D) (16, 24) and the resulting effector to target (E:T ratio) is therefore much higher with α -CD3-CD19 and α -C1-CD19 than with α -V5-CD19 and α -V12-CD19. Increasing the E:T ratio by co-culturing NALM6 B cells with TRBV5⁺ or TRBV12⁺ enriched T cells in the presence of α -V5 or α -V12 resulted in robust IFN γ production (Fig. 2G) and NALM6 B cell cytotoxicity (Fig. 2H). This suggests that the low E:T ratio was responsible for the relatively low cytokine production and NALM6 B cell cytotoxicity observed with α -V5-CD19 and α -V12-CD19 (Fig. 2E and F).

We performed similar experiments that demonstrated α -C1 could mediate the death of clonal, neoplastic T cells expressing TRBC2 through bidirectional killing, even though these neoplastic T cells did not express TRBC1 (fig. S4, A to D). α -C1 exposure induced IFN γ production against both TRBC1⁺ (Jurkat) and TRBC2⁺ (HPB-ALL) cells (fig. S4C). Depletion of TRBC1⁺ T cell subset limited α -C1 induced IFN γ production against HPB-ALL cells (fig. S4C). Flow cytometry analysis also showed α -C1 mediated HPB-ALL cell death with

the use of total T cells, while TRBC1⁺ T cell depletion reversed the effect (fig. S4D). Depletion of normal TRBC1⁺ T cells did not affect α -C1 induced IFN γ response to Jurkat cells (fig. S4C) or α -C1 mediated Jurkat cell killing (fig. S4D) as α -C1 activated the remaining normal TRBC2⁺ T cells by crosslinking of CD3 on these cells with TRBC1 on Jurkat cells. We concluded that potent bidirectional killing can be mediated by BsAbs targeting TRBC1, TRBV5-5, or TRBV12, but the reduced frequency of TRBV5-5⁺ or TRBV12⁺ T cells results in less bidirectional killing as compared to TRBC1⁺ T cells.

TRBV-directed BsAbs induce T cell cytokine responses against cancer cells in vitro

Human T-cell cancer-derived cell lines have rearranged TCR β genes and express clonal TRBVs (38). We observed that T-ALL derived Jurkat, HPB-ALL and CCRF-CEM T cell lines retained cell-surface TCR expression as assessed with anti-CD3 antibodies, whereas MOLT3 cells did not (fig. S5A) (39). Jurkat and HPB-ALL cells also expressed surface TRBV12 and TRBV5-5, respectively (fig. S5B), as expected (38, 40). To assess the activity of BsAbs against T-cell malignancies, we co-cultured normal T cells with T-cell cancer cell lines in the presence or absence of different BsAbs. An increase in baseline IFN γ production, in the absence of any cancer cells, was noted after exposure to α -C1 ($p < 0.0001$) and to a lesser degree with α -V5 and α -V12 (Fig. 3A). α -V5 and α -V12 increased T cell IFN γ secretion above baseline in the presence of HPB-ALL (TRBV5-5⁺, $P < 0.0001$) and Jurkat (TRBV12-3, $P < 0.0001$) cells, respectively. To confirm that the baseline IFN γ production in the absence of target cancer cells was a result of the small percentage of TRBV5-5⁺ and TRBV12⁺ T cells present in the normal T cells, we depleted these cells before exposure to the BsAbs. As expected, TRBV5- and TRBV12-depleted T cells failed to produce IFN γ in response to α -V5 and α -V12, respectively (Fig. 3B). As a control for this experiment, we showed that the depletion of TRBV5-5⁺ and TRBV12⁺ T cells did not affect IFN γ production in the presence of the α -C1 BsAb (Fig. 3B). Additionally, the TRBV5-depleted T cells secreted IFN γ when co-cultured with HPB-ALL (TRBV5-5⁺) cells in the presence of α -V5. Similarly, TRBV12-depleted T cells co-cultured with Jurkat (TRBV12-3⁺) cells in the presence of α -V12 also secreted IFN γ . This also indicated that the TRBV depletion process itself did not result in loss of normal T cell function. The T cell activation via α -V5 and α -V12 was poly-functional, demonstrated by the release of multiple cytokines including TNF- α , IL-2, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in addition to IFN γ (Fig. 3, C and D).

As a further control for specificity of these BsAbs, we created isogenic cancer cells using CRISPR-based disruption of TCR alpha and beta constant regions in both Jurkat and HPB-ALL cell lines. The resultant TCR knock-out (KO) was confirmed by loss of cell-surface TRBV12 or TRBV5-5 (fig. S5B).

After TCR-KO, only a small increase in IFN γ or four other cytokines tested was observed upon co-culturing HPB-ALL cells with normal T cells in the presence of α -V5 (Fig. 3C). Similarly, minimal increase in cytokines was observed after co-culturing TCR-KO Jurkat cells with normal T cells in the presence of α -V12 (Fig. 3D).

TRBV-directed BsAbs kill cancer cell lines in vitro

To assess cytotoxicity, we co-cultured normal human T cells with Jurkat or HPB-ALL cells in the presence of increasing concentrations of α -V12 or α -V5 BsAbs (Fig. 4A, B). We observed almost complete Jurkat and HPB-ALL cytotoxicity at 0.01 nM (0.57 ng/mL) concentration of α -V12 and α -V5. We also engineered cancer cell lines to express GFP. Jurkat cells expressing GFP were eliminated when co-cultured with normal T cells in the presence of α -V12 (Fig. 4C, D and fig. S6A). Exposure to α -V12 and normal T cells had no significant effect on TCR-KO Jurkat cells ($P = 0.84$, Fig. 4C, D and fig. S6A). Similarly, HPB-ALL cells expressing GFP were eliminated when co-cultured with normal T cells in the presence of α -V5, and this elimination was abrogated in TCR-KO HPB-ALL cells (Fig. 4E, F and fig. S6B). As another control for this experiment, we showed that the α -CD19 BsAb had no effect on either Jurkat or HPB-ALL cells when incubated with normal T cells (Fig. 4, C to F and fig. S6A and B). α -V12 also induced expression of activation and exhaustion markers on the normal human T cells in the presence of the target Jurkat cells (fig. S6C). Similarly, α -V5 mediated expression of activation and exhaustion markers on the normal human T cells in the presence of the target HPB-ALL cells (fig. S6D). We did not observe a loss of α -V12 and α -V5 activity with depletion of the CD4 helper T cells from normal human T cells (fig. S6E and F). Additionally, α -V12 and α -V5 cytotoxic function was preserved after incubation of the BsAbs with human serum for 96 hours prior to co-culture (fig. S6G).

To determine whether α -V12 affects T cells expressing TRBV-families other than TRBV12, we co-cultured Jurkat cells and normal T cells in the presence of α -CD19 or α -V12. We then performed TRBV gene sequencing to measure the percentage of TRBV depletion in surviving cells. As expected, we detected a dramatic reduction (98.9%) in the proportion of TRBV12-3 of total TRBV signal after exposure to α -V12 compared with exposure to α -CD19 (fig. S7A). The vast majority of the TRBV12-3 signal was derived from the Jurkat cells rather than the normal T cells. We also noticed a reduction in TRBV12-4 by 36.5%, but the difference was not statistically significant ($P = 0.22$) (fig. S7A). All other TRBV family members were unaffected (fig. S7A). A similar analysis was performed with HPB-ALL cells. As expected, we detected a dramatic reduction (98.3%) in the proportion of TRBV5-5 of total TRBV signal after exposure to α -V5 compared to that after exposure to α -CD19 (fig. S7B). The vast majority of the TRBV5-5 signal was derived from the HPB-ALL cells rather

than the normal T cells. Again with the exception of TRBV5-6, which was reduced by 91.6% ($p < 0.0001$, fig. S7B), other TRBV family members remained unaffected. The sequence of the TRBV5-5 directed scFv we used in our α -V5 BsAb was derived from an antibody originally developed against a TRBV5-5 antigen (41). Thus it was not surprising that TRBV5-6-expressing T cells were affected by α -V5 exposure given that TRBV5-5 and TRBV5-6 are the most similar among TRBV5 family members (fig. S8A, B). Sequence alignment of TRBV5 family members revealed that amino acid residues D20, D81, and L101 are common to both TRBV5-5 and TRBV5-6 but differ from other TRBV5 members, and the differences at residues 81 and 101 in the other TRBV5 family members also resulted in major charge differences (fig. S8C).

TRBV-directed BsAb kill patient-derived T-ALL cells in vitro

We collected primary malignant cells from T-ALL patients. Flow cytometry identified two patients (Patients 1 and 2) with a substantial TRBV12⁺ population, suggesting presence of monoclonal cancer cells (Fig. 5A). T-ALL cells from patients 1 and 2 co-cultured with normal T cells in the presence of α -V12 led to significant IFN γ secretion ($P < 0.0001$, Fig. 5B), and expression of activation and exhaustion markers on normal human donor T cells (Fig. 5C). We used HLA-A3 expression to discriminate between the normal T cells derived from two healthy human donors and patient-derived T-ALL cells (Fig. 5D). Co-culture of Donor-2 T cells (HLA-A3⁺) with Patient-1 (HLA-A3⁻) malignant cells and α -V12 showed depletion of patient-derived malignant cells ($P < 0.0001$) (Fig. 5E, F). Similarly, co-culture of Donor-1 (HLA-A3⁻) T cells with Patient-2 (HLA-A3⁺) malignant cells also showed depletion of the malignant cells ($p < 0.0001$). In both cases, the normal human T cells were relatively unaffected by exposure to α -V12 (Fig. 5E, F), as expected from the low fraction of TRBV12-expressing cells among normal T cells (Fig. 1C, D).

TRBV-directed BsAbs kill cancer cells in vivo

To assess efficacy in vivo, we established two disseminated xenograft models with luciferase-expressing Jurkat or HPB-ALL cancer cells injected intravenously into NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice (Fig. 6, A to C). All mice also received human T cells via intravenous injection. For the Jurkat model, the α -V12 BsAb was delivered through an intraperitoneal pump starting on day 4 after Jurkat and normal human T cell infusion, when Jurkat cells were already widely disseminated (Fig. 6, A and B). The intraperitoneal pumps were able to maintain consistent serum concentrations of α -V12 and α -V5 for at least 2 weeks after implantation (fig. S9A). Bioluminescence imaging (BLI) demonstrated marked tumor burden reduction in the mice treated with α -V12 (Fig. 6, B and D). Two controls were used to document the specificity of this reduction, one for the BsAb and one for the cells.

For the BsAb control, mice harboring Jurkat cancers were treated with α -CD19 instead of α -V12. In this case, tumor burden was significantly higher as assessed by BLI ($P < 0.0001$, Fig. 6, B and D). For the cell control, mice bearing disseminated cancers derived from Jurkat TCR-KO cells were compared to mice bearing WT Jurkat cells. We demonstrated that tumor burden was markedly higher compared to mice bearing WT Jurkat cells after treatment with α -V12 ($P = 0.0369$, Fig. 6, B and D). A second disseminated cancer model was used to document reproducibility of these in vivo results. The experimental approach was identical to that described for the Jurkat cell model, except that HPB-ALL cells were substituted for Jurkat cells and α -V5 was substituted for α -V12 (Fig. 6, A and C). Again BLI demonstrated marked luminescence reduction in the mice treated with α -V5 ($P = 0.01$, Fig. 6, C and D) when compared to α -CD19 treatment. Mice bearing disseminated cancers derived from HPB-ALL TCR-KO cells also demonstrated a tumor growth similar to observations in the Jurkat model ($P < 0.0001$, Fig. 6, C and D). Nineteen days after inoculation of cancer cells (15 days after initiating BsAb treatment) flow cytometric analysis of mouse blood revealed that all treatment groups retained normal human T cells (Fig. 6, E and F). Additionally, there were abundant circulating Jurkat and HPB-ALL leukemia cells in α -CD19 treated mice (Fig. 6, E and F). In striking contrast, α -V12 and α -V5 treated mice had a dramatic reduction in circulating leukemia cells in these experiments ($P = 0.01$ and $P < 0.0001$, Fig. 6, E and F). This reduction in circulating leukemia cells was associated with a significant survival benefit in both α -V12 and α -V5 treated mice ($P < 0.0001$, Fig. 6, G and H). α -CD19 treated mice developed hind-leg paralysis (Video S1) as previously noted with disseminated Jurkat xenograft models (42), leading to the need for euthanasia. Mice bearing Jurkat or HPB-ALL cancers that were treated with α -V12 or α -V5, respectively, did not develop hind-leg paralysis. These mice were eventually euthanized (Fig. 6, G and H) when they began to demonstrate typical graft-versus-host-disease (GVHD) features. Xenogenic GVHD was expected based on prior reports (16, 43, 44) and is a result of human T cell transfer into mice.

In human T-cell cancers, an additional challenge is that the malignant T cells often outnumber the healthy effector T cells. To ascertain if a lower number of human effector T cells can sufficiently eradicate the T-cell tumors in vivo, we injected NSG mice with 0.5×10^6 human T cells along with 2.5×10^6 tumor cells (Jurkat or HPB-ALL cells) (fig. S9B). BLI demonstrated significant tumor burden reduction with both α -V12 and α -V5 treatment ($P = 0.038$ and $P = 0.001$, fig. S9C to E). α -V12 and α -V5 treatment also lead to elevated IFN γ and TNF α cytokine production ($P < 0.0001$, fig. S9F, G) along with expression of T cell activation and exhaustion markers on normal human T cells ($P = < 0.0001$, fig. S9H, I).

DISCUSSION

Clonally rearranged cell-surface receptors have been exploited in the past as immunotherapy targets. Studies by the groups of Ronald Levy (45) and George Stevenson (46) pioneered the use of patient-specific anti-idiotypic antibodies to selectively target cancerous B cells. However such bespoke anti-idiotypic antibodies need to be generated for individual patients and these strategies were superseded by pan-B cell targeting antibodies and CAR T cells with demonstrable safety profiles (8–10, 12–14, 47). Pan-B cell targeting was also enabled by the availability of donor derived immunoglobulin infusions to substitute for B cell function in patients suffering from B cell aplasia-induced immunosuppression (48). Current investigational T-cell immunotherapies target pan-T cell antigens including CD4 (49), CD5 (50), and CD7 (51) in CAR T cell formats. These CAR T strategies require additional cellular engineering to block target antigen expression by the CAR T cells to prevent CAR T cell fratricide. Additionally, mitigation strategies analogous to immunoglobulin infusions in B cell deficiencies are not available to substitute for the loss of T cell function.

Another innovative strategy to target T-cell leukemias employs TRBC1-targeting-CARs. These destroy approximately half of the T-cell repertoire and thus preserve a functioning immune system (16). However, the CAR targeting format is associated with logistical hurdles, involving autologous CAR T generation and deployment, which is a time- and resource-intensive process (27). BsAbs provide an off-the-shelf alternative therapeutic option to CAR T cells. An early phase clinical trial demonstrated BsAb activity in CAR T cell refractory B cell lymphoma patients (9), supporting the notion that BsAbs provide potential efficacy, cost and logistical benefits over CAR T cells. On the other hand, the single infusion of CAR T cells provides a distinct advantage over the need for continuous BsAb therapy and either approaches might optimally serve different patient populations.

We hypothesized that α -C1 BsAb would offer an alternate, easier-to-adopt strategy compared to CAR T cell targeting of cancer cells expressing TRBC1. Unfortunately, bidirectional T cell killing with α -C1 caused near complete depletion of TRBC1 and TRBC2 T cells, making such an approach unfeasible. Alternate antibody-based therapeutic formats, such as anti-TRBC1 antibody drug conjugates (ADCs), could potentially be adopted to circumvent bidirectional killing and limit healthy T cell depletion, albeit with a risk of dose limiting toxicities (52, 53). Bidirectional killing, however, was not reported with TRBC1 targeting CAR study (16) and may reflect the faster killing kinetics of CAR T cells (54), allowing the CAR T cells to escape donor T cell-mediated killing. BsAbs are unlikely to generate cancer-specific memory T cells because the antigens are only indirectly recognized by the T cells through the CD3 moiety, rather than through their

endogenous TCRs. It is interesting to note that CD4 and CD8 T cells with memory like phenotypes were detected in blinatumomab-treated patients with B cell malignancy (55, 56). However, it is known whether the presence of these memory T cells correlate with therapeutic response or if they are essential for long term disease remission. In view of these uncertainties, in our pre-clinical assessment of the TRBV-targeting BsAbs, we evaluated the direct tumor cell killing properties in vitro and in vivo.

Anti-idiotypic targeting required a unique monoclonal antibody generation for each B cell cancer patient. TRBV targeting for T-cell cancers would require development of approximately 30 BsAbs to target all TRBV families. This is feasible, as 24 of the 30 required TRBV targeting scFvs are already available (24) and can be re-engineered to serve as BsAbs. A large study involving more than three hundred CTCL patients revealed TRBV20 as the most common cancer-associated TRBV, confirming a previous report (31, 32). Further, 10 TRBV families (TRBV20, TRBV21, TRBV7, TRBV5, TRBV6, TRBV3, TRBV10, TRBV28, TRBV19, and TRBV12) collectively constitute about 79% of all the TRBVs found in CTCL. Thus it may be possible to target the majority of cancer-associated TRBVs with a more limited set of anti-TRBV BsAbs.

We expect that TRBV targeting for cancer treatment will encounter certain predictable limitations. Therapeutic TRBV or TRBC targeting may lead to CD3 or TCR negative relapses based on prior clinical experience with single antigen targeting biologics (57–59). TRBV targeting may also generate toxicity, such as increased susceptibility to infections if the targeted TRBVs are required to respond to particular pathogens (60–62), although functional redundancy among non-targeted TRBV families may limit such effects. Bidirectional killing can also trigger cytokine release syndrome, and the removal of regulatory T cells with specific TRBV predilection may interfere with immune tolerance (63). Additionally, variations in patients' effector T cell functions may lead to heterogeneous anti-tumor clinical responses (64–66). We found that BsAb mediated targeting of the TCR clones in healthy donor T cells results in activation of specific T cell subsets. This raises the possibility of potential tumor T cell proliferation as a result of TRBV-targeting BsAb engagement. We did not find evidence of tumor T cell expansion in our in vitro and in vivo models, confirming previous reports of the absence of T-cell tumor proliferation through targeting of TRBC1 on cancer cells (16). Ultimately, the feasibility and toxicity of TRBV targeting will need to be established through clinical trials.

Our study also carries implications outside the field of T-cell malignancies. We have recently demonstrated that bispecific antibodies, designed precisely as described herein, can kill cancer cells expressing common mutations in TP53

(67) or RAS (68) that are presented in complex with common HLA types. Additionally, the results reported here show that that TRBC or TRBV engagement induces T cell activation and target cell death. Current BsAbs utilize α -CD3 for global T cell activation against target cells, and in the process can trigger an immunologic cascade leading to deadly cytokine release syndrome (69). α -TRBV-mediated harnessing of unique T cell subpopulations as anti-cancer effector cells, could limit the toxicities associated with pan-T cell activation and lead to safer therapies. Selective T cell clonal expansions have been detected in GVHD (70, 71), the primary complication of allogeneic bone marrow transplantations. Furthermore, animal studies suggest that selective TRBV depletion can induce tolerance to the allograft (72, 73). Certain autoimmune pathologies such as celiac disease are also driven by specific TRBV families (74–76). TCR sequencing to identify the disease causing TRBV clones followed by TRBV-directed depletion of these T cells could open up new therapeutic strategies for GVHD and celiac disease management.

We demonstrate that our bispecific antibodies targeting TRBV12 and TRBV5-5 selectively depleted malignant T cells both in vitro and in animal models of T-cell cancers. Additionally, our TRBV-directed therapies preserved the vast majority of healthy human T cells. Thus future management of clonal T cell driven pathologies may involve TCR sequencing to identify the disease causing T cell clone followed by TRBV targeting antibody therapy to specifically eradicate the offending T cell population while preserving a functioning cellular immune system.

MATERIALS AND METHODS

Study design

The study goal was to determine the selective T cell targeting potential for TRBV-directed antibodies in human T-cell malignancies. We utilized anti-TRBV5-5 and anti-TRBV12 scFv sequences to generate bispecific antibodies α -V5 and α -V12 respectively. Both T-cell cancer cell lines and patient derived T-cell cancers were used to test α -V5 and α -V12 bispecific antibody activity. Primary human T cells obtained from healthy human donors were used as effector cells. The T-cell cancer patient samples were collected in accordance with the Johns Hopkins Institutional Review Board (IRB: NA_00028682, and NA_00028682) approved Hematologic Malignancy Cell Bank Protocol (J0969) or the Johns Hopkins Pediatric Leukemia Bank Protocol (J0968). The selective T-cell cancer killing potential of α -V5 or α -V12 was studied using in vitro co-cultures. T cell activation was measured using cytokine-specific ELISA and Luminex assays. Cytotoxicity was estimated using luciferase activity and flow cytometry based detecting of different cell populations. For all animal experiments, mice were injected with tumor cells followed by randomization into different treatment groups. The

experimenters were not blinded. Sample sizes for animal experiments were selected based on previous experience with the animal models but were not pre-determined by power analysis. No animals were excluded because of illness from the study. The number of replicates in each experiment are noted in the figure legends.

Cell lines and primary human T cells

Jurkat (Clone E6-1), CCRF-CEM, MOLT-3, (American Type Culture Collection, (ATCC)), HPB-ALL (Deutsche Sammlung von Mikroorganismen und Zellkulturen, (DSMZ)) and NALM6 (kind gift from Martin Pomper and Il Minn) (77) were cultured in RPMI-1640 (ATCC, 30-2001) supplemented with 10% HyClone fetal bovine serum (FBS, GE Healthcare SH30071.03) and 1% Penicillin-Streptomycin (ThermoFisher Scientific). HEK293FT (ThermoFisher Scientific) was cultured in DMEM (ThermoFisher Scientific, 11995065) supplemented with 10% FBS, 2mM GlutaMAX (ThermoFisher Scientific, 35050061), 0.1mM MEM non-essential amino acids (ThermoFisher Scientific, 11140050), 1% Penicillin-Streptomycin, and 500 μ g/mL Geneticin (ThermoFisher Scientific, 10131027). Peripheral blood mononuclear cells were isolated from leukapheresis samples (Stemcell Technologies, Celler) by Ficoll Paque Plus (GE Healthcare, GE17-1440-02) density gradient centrifugation. Human T cells were expanded from peripheral blood mononuclear cells either with addition of the anti-human CD3 antibody (clone OKT3, BioLegend) at 15 ng/mL, or with Human T-Activator CD3/CD28 Dynabeads (ThermoFisher Scientific, 11131D) for three days at a bead:cell ratio of 1:5. T cells were cultured in RPMI-1640 with 10% FBS, 1% Penicillin-Streptomycin, 100 IU/mL recombinant human IL-2 (aldesleukin, Prometheus Therapeutics and Diagnostics), and 5 ng/mL recombinant human IL-7 (BioLegend, 581906).

Cell staining, flow cytometry and cell sorting

Cells were suspended at 1×10^6 cells/mL in flow stain buffer composed of phosphate buffered saline (PBS) and 0.5% BSA, 2 mM EDTA, 0.1% sodium azide or flow sorting buffer (PBS containing 4% FBS) and incubated with appropriate antibodies at a concentration of 1 μ g/mL for 30 min on ice, in the dark. The antibodies used were: Brilliant Violet (BV)-711 anti-human CD3 (clone OKT3 BioLegend, #317328); allophycocyanin (APC)-anti-human CD45 (clone HI30 BioLegend, #304012); APC-anti-human CD19 (clone HIB19, Biolegend, #302212), phycoerythrin (PE)-anti-human CD4 (clone RPA-T4, Biolegend, #300508), APC-anti-human CD8 (clone SK1, Biolegend, #344722) PE-anti-human C β 1 TCR (clone JOV1.1 BD Biosciences, #565776); PE-anti-human HLA-A3 (clone GAP.A3 BD Biosciences, #566605); PE-TCR $\nu\beta$ 5.1 (clone ImmU157, Beckman Coulter); PE-TCR $\nu\beta$ 5.3 (clone 3D11, Beckman Coulter), PE-TCR $\nu\beta$ 5.2 (clone 36213, Beckman Coulter), fluorescein isothiocyanate (FITC)-TCR $\nu\beta$ 8 (clone 56C5.2 Beckman Coulter), BV-421-anti-human CD25 (clone

BC96, Biolegend, #302630), APC-anti-human ICOS (C398.4A, Biolegend, #313510), BV-750-anti-human-41BB (clone 4B4-1, Biolegend# 309844), BV-421-anti-human LAG3 (clone 11C3C65, Biolegend# 369314), APC-anti-human-PD1 (clone EH12.2H7, Biolegend, #329908). Stained cells were analyzed using an LSRII flow cytometer or sorted using BD FACSAria II (Becton Dickinson). Gating on single live cells was performed with the use of viability dyes (LIVE/DEAD Fixable Near-IR, L10119; Aqua Dead Cell Stain Kit L34957 Invitrogen) and forward and side scatter characteristics. CellTrace Violet stain (ThermoFisher C34557) was performed per manufacturer instructions.

TRBC, TRBV and CD4 depletion or enrichment

For TRBC1 T cell depletion, 1×10^8 normal T cells were stained with PE-mouse anti-human C β 1 TCR (final concentration 1 μ g/ml) followed by PE negative (TRBC1-depleted) cell sorting. For TRBV5 T cell depletion or enrichment, 1×10^8 normal T cells were stained with PE-TCR V β 5.3 (binds TRBV5-5) and PE-TCR V β 5.2 (binds TRBV5-6), followed by PE negative (TRBV5-depleted) or PE positive (TRBV5 enriched) cell sorting. For TRBV12 T cell depletion or enrichment, 1×10^8 normal T cells were stained with FITC-TCR V β 8 (binds TRBV12-3 and TRBV12-4 T cells), followed by FITC negative (TRBV12-depleted) or FITC positive (TRBV12 enriched) cell sorting. Alternatively, an EasySep PE Positive Selection Kit II (Stemcell Technologies, 17684) was used for cell isolation per manufacturer's instructions. For CD4 T cell depletion, normal T cells were stained with PE-anti-human CD4 followed by EasySep PE Positive Selection Kit II used for CD4 negative (CD4-depleted) cell isolation.

Bispecific antibody production, purification and stability

The α -TRBV5-5, α -TRBV12, α -TRBC1 and α -CD19 scFv sequences (table S1) were synthesized by GeneArt (ThermoFisher Scientific). All scFv sequences have been previously published or patented. The K_d values in table S1, were previously reported using Biacore assay (α -TRBC1) or using Scatchard analysis (α -TRBV5-5, α -TRBV12). The scFv sequence was expressed as single chain diabody format using the following N- to C terminus format: IL-2 signal sequence, anti-TRBV/TRBC/CD19 variable light chain (VL), GGGGS linker, α -CD3 variable heavy chain (VH), (GGGGS) $_3$ linker, α -CD3 VL, GGGGS linker, anti-TRBV/TRBC/CD19 VH, and 6 x HIS tag, and cloned into a pcDNA3.4 vector (ThermoFisher Scientific). BsAbs were expressed and purified by the Johns Hopkins University (JHU) Eukaryotic Tissue Culture Core Facility or by GeneArt. For BsAb expression from JHU Eukaryotic Tissue Culture Core Facility, 1 mg of plasmid was transfected with polyethylenimine (PEI) at a ratio of 1:3 into a 1 L suspension culture of HEK293F cells at a density of 2×10^6 cells/mL. Transfected HEK293F cells were grown in FreeStyle293 expression media for 5 days at 37°C, 170 rpm, and

5% CO $_2$. Subsequently, the media was harvested by centrifugation, filtered with a 0.22 μ m unit and the BsAb was purified using Nickel affinity chromatography. For this purpose, 2 mL of Ni-NTA His-Bind (Millipore Sigma, 70666-6) resin was added to the filtered supernatant and incubated at 4°C overnight in an orbital shaker. The supernatant-resin mixture was captured by a gravity chromatography column (Econo-Pac Chromatography Columns 7321010, Bio-Rad) and washed with 20 mM imidazole (GE Healthcare, 45-000-007) in PBS. The desired BsAb was eluted with 500mM imidazole and desalted into PBS using a 7k MWCO Zeba Spin desalting column (ThermoFisher Scientific, 89883). Proteins were quantified via SDS-PAGE gel electrophoresis (Mini-PROTEAN TGX Stain-Free Precast Gel, Bio-Rad, 4568095) or using bicinchoninic acid (BCA) protein assay (Pierce, ThermoFisher, 23225). Proteins were stored at -80°C with 7% glycerol. Alternatively, BsAbs were produced by GeneArt in Expi293s, and purified with a HisTrap column (GE Healthcare, 17-5255-01) followed by size exclusion chromatography using a HiLoad Superdex 200 26/600 column (GE Healthcare, 28989336). Analytic chromatography was performed using TSKgel G3000SWxl column (TOSOH Bioscience) using a running buffer of 50 mM sodium phosphate and 300 mM sodium chloride at pH 7 at a flow rate of 1.0 mL/min. Coomassie blue stain (ThermoFisher Scientific, 20278) of sodium dodecyl sulfate gel and anti-histidine Western blot with anti-6x-His tag antibody (ThermoFisher Scientific, MA1-21315) was used to evaluate the purity of BsAb by GeneArt. Thermal stability of the α -V12 and α -V5 BsAbs were evaluated by a differential scanning fluorimetry, which monitors the fluorescence of a dye that binds to the hydrophobic region of a protein as it becomes exposed upon temperature induced denaturation (78). Reaction mixtures (20 μ L) were set up in a white low-profile 96-well, unskirted polymerase chain reaction plates (Bio-Rad, MLL9651) by mixing 2 μ L of purified α -V12 or α -V5 BsAb at a concentration of 1 mg/mL with 2 μ L of 50X SYPRO orange dye (Invitrogen S6650) in pH 7.4 PBS (Gibco, 10010023). Plates were sealed with an optical transparent film and centrifuged for $1000 \times g$ for 30 s. Thermal scanning was performed from 25 to 100°C (1°C/min temperature gradient) using a CFX9 Connect real-time polymerase chain reaction instrument (Bio-Rad). Protein unfolding and melting temperature (T_m) was calculated from the maximum value of the negative first derivative of the melt curve using CFX Manager Software (Bio-Rad). Serum stability was assessed by incubating the BsAbs with human serum (Millipore Sigma #H4522) at 0.05 μ g/mL concentration in a 37°C incubator for 0, 24 and 96 hours. At each time point, the human serum BsAb mixture was collected and frozen at -80°C until BsAb functional analysis by a co-culture assay.

CRISPR gene editing

The Alt-R CRISPR system (Integrated DNA Technologies)

was used to generate TCR knock-out Jurkat and HPB-ALL cell lines, as well as CD19 knock-out and CD19 low expressing NALM6 clones. For the knockout of TCRs, Alt-R CRISPR Cas9 crRNAs targeting the TRA constant region (AGAGTCTCTCAGCTGGTACA), TRB constant region (AGAAGGTGGCCGAGACCCTC), and Alt-R CRISPR-Cas9 tracrRNA (IDT, 1072533) were re-suspended at 100 μ M in Nuclease-Free Duplex Buffer (IDT, 11-01-03-01). The crRNAs and tracrRNA were duplexed at a 1:1 molar ratio for 5 min at 95°C followed by cooling down slowly to room temperature according to the manufacturer's instructions. The duplexed RNA was then mixed with Cas9 Nuclease at a 1.2:1 molar ratio for 15 min. A total of 40 pmoles of the Cas9 ribonucleoprotein complexed with gRNA were mixed with 500,000 cells in 20 μ L of OptiMEM (ThermoFisher, 51985091). This mixture was loaded into a 0.1 cm cuvette (Bio-Rad) and electroporated at 90 V for 15 ms using an ECM 2001 (BTX). Cells were immediately transferred to complete growth medium and cultured for 7 days. Single cell clones were established by limiting dilution and genomic DNA isolated using a Quick-DNA 96 Kit (Zymo Research, D3010). Regions flanking the CRISPR cut sites were PCR amplified (TCR α forward primer: GCCTAAGTTGGGGAGACCAC, reverse primer: GAAGCAAGGAAACAGCCTGC; TCR β forward primer: TCGCTGTGTTTGAGCCATCAGA, reverse primer: ATGAACCACAGGTGCCCAATTC) and Sanger sequenced to select for TCR α -/ β - clones. To generate CD19 knock-out and CD19 low NALM6 clones, an Alt-R CRISPR sgRNA (CGAGGAACCTCTAGTGGTGA) was complexed with Cas9 Nuclease (IDT) at a 2:1 molar ratio for 15 min at room temperature. Then, 50 pmoles of Cas9 RNP were mixed with 200,000 NALM6 cells re-suspended in 20 μ L SF buffer (Lonza) and electroporated with a 4D Nucleofector X-unit (Lonza) in 16-well cuvette strips using pulse code CV-104. The cells were cultured in complete growth media for 7 days prior to dilutional plating to select individual clones. Cell surface CD19 expression of clones were characterized by flow cytometry staining with anti-human CD19 antibody.

Retroviral transduction

Non-tissue culture treated plates were coated with 100 μ L RetroNectin (Clontech Takara, T202) in PBS at 20 μ g/mL overnight at 4°C, then blocked with 10% FBS for 1 hr at room temperature. Retrovirus (RediFect Red-FLuc-GFP, PerkinElmer CLS960003) and 2×10^5 target cells were added to each well and centrifuged at $2000 \times g$ for 1 hr at 20°C. Plates were incubated for two days at 37°C, after which cells were expanded to a 6-well plate. Transduced cells were sorted using a BD FACSAria II based on GFP expression.

TCR sequencing

Total RNA was isolated from samples with Qiagen AllPrep DNA/RNA Micro kits (Qiagen, 80284). RNA quality was validated using an Agilent TapeStation system. TCR sequencing

libraries were prepared using a 5' RACE (rapid amplification of cDNA ends) method consisting of a cDNA synthesis step followed by two PCR steps with gene-specific primers for the TCR β constant region. Libraries were sequenced using an Illumina MiSeq platform. Reads were analyzed with MIGEC, MiXCR, and VDJtools (79–81). Frequencies of clonotypes were calculated as the proportion of UIDs (unique molecular identifier barcodes) representing the clonotype among all UIDs in the sample. The following non-functional TRBVs (listed as pseudogenes or as open reading frames in the international ImMunoGeneTics information system (IMGT) database) were excluded from analysis: TRBV1, TRBV3-2, TRBV5-2, TRBV5-3, TRBV5-7, TRBV6-7, TRBV7-1, TRBV7-5, TRBV8, TRBV12-1, TRBV12-2, TRBV21, TRBV22, TRBV23-1, TRBV26.

TRBV sequence and structural alignment

The structures of PDB ID 5BRZ (82), 6EH5 (83), 4P4K (84) and 4QRR (85) were structurally aligned and residues 2-95 were extracted from 5BRZ, corresponding to the TCR beta variable region of TRBV 5.1. To model TRBV 5.4, 5.5, 5.6 and 5.8, in silico mutations were performed at positions 81 and 101 using Coot (86). Figures were rendered in PyMOL (v2.2.3, Schrödinger, LLC). Alignment of relevant TRBV sequences was performed using ClustalOmega (87) and displayed using Espright (88).

Co-cultures

Co-cultures were set up using 96-well flat-bottom tissue culture treated plates, with each well containing 5×10^4 normal human T cells (effector cells), 5×10^4 target cells (indicated in text) and BsAbs (concentration specified in text) in a total 100 μ L volume RPMI media. The co-cultures were incubated for 17 hours at 37°C. The supernatant was assayed for cytokines using a Human IFN-gamma Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, SIF50C), Human IL-2 Quantikine ELISA Kit (R&D Systems, S2050), Human TNF-alpha Quantikine ELISA Kit (R&D Systems, STA00D), Human IL-10 Quantikine ELISA Kit (R&D Systems, S1000B) or a Luminex assay (13-plex -Immunology Multiplex Assay, Millipore Sigma, USA, HMHEMAG-34K) performed on the BioPlex 200 system (Bio-Rad). For luciferase expressing target cells, cell viability was assayed by the Steady-Glo luciferase assay (E2510, Promega), per manufacturer's instructions. Viability was calculated as the ratio of luminescence signal to the no antibody or control antibody condition: (antibody well luminescence)/(no antibody or control antibody well luminescence). Alternatively, tumor cells were quantified by flow cytometry based GFP expression (for GFP-expressing tumor cell lines) or distinct HLA expression (for patient-derived tumor cells). For experiments to detect effects of BsAbs on healthy T cells in the absence of target tumor cells, 1×10^6 normal human T cells was incubated with the BsAbs (concentration specified in text) in a total 1 mL volume RPMI media

for 17 hours at 37°C. Viable T cells were quantified by counting trypan blue stained cells on a hemocytometer.

Animal experiments

Six to eight week old female NOD.Cg-*Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ* (NSG) mice acquired from the Johns Hopkins Sidney Kimmel Comprehensive Cancer Center Animal Resources facility were maintained according to JHU Animal Care and Use Committee approved research protocol MO18M79. Cancer cell lines and human T cells were injected via the tail vein as indicated. Two-week micro-osmotic pumps (Model 1002, ALZET) were filled with BsAb using a 30G needle. Pumps were placed in the peritoneal space of each mouse using sterile surgical technique. Briefly, after the animal was anesthetized, the skin over insertion site was shaved and sterilized. A 1.5 cm midline incision was made over the lower abdomen. The underlying peritoneum was incised and the pump was inserted into the peritoneal cavity. The peritoneal layer was closed using absorbable sutures, and the skin incision was closed using wound clips. Wound clips were removed once the incision site has healed, usually around day 10 after surgery. For survival studies, animals were followed until day 80 or euthanized when evidence of paralysis or GVHD was observed (hunched posture, fur ruffling, scaling or denuded skin, reduced activity). Mouse bioluminescence was measured using the IVIS system (PerkinElmer). Prior to imaging, mice were anesthetized using inhaled isoflurane in an induction chamber. Following induction, mice received intraperitoneal injection of luciferin (150 μ l, RediJect D-Luciferin Ultra Bioluminescent Substrate, PerkinElmer, 770505) and were placed in the imaging chamber after 5 min. Luminescence images were analyzed using Living Image software (version 4.7.2, PerkinElmer). For flow-based detection of tumor cells and normal human T cells from mouse blood, 100 μ L blood was collected in EDTA treated microvettes (Sarstedt Inc, NC9299309) by mouse cheek bleed, followed by 10 min incubation with 1 mL ACK lysis buffer (Quality Biological, 118-156-721), resuspension in flow stain buffer with mouse and human TrueStain FcX Fc receptor blocking solutions (BioLegend, 101320, 422302) and cell-surface staining antibodies. 10 μ L of counting beads (Precision Count Beads, BioLegend, 424902) were added to equal volume (300 μ L) of cell suspension in each tube. The number of tumor cells (GFP+, CD3+) or T cells (GFP-, CD3+) were counted based on acquisition of 500 beads for each sample. For cytokine and BsAb detection, blood from mice was collected in Eppendorf tubes and allowed to clot for 30 min at room temperature, followed by centrifugation at 1000 x g for 5 min at 4°C. Serum was collected and stored at -80°C until a cytokine enzyme-linked immunosorbent assay (ELISA, per manufacturer instructions) or a BsAb ELISA was performed. For BsAb ELISA, mouse serum was incubated in biotinylated recombinant human CD3 epsilon & CD3 delta (Acro Biosystems, # CDD-

H52W4) coated streptavidin plates (R&D Systems, #CP004), followed by detection using HRP conjugated anti-human kappa light chain antibody (ThermoFisher Scientific, #A18853).

Statistical analyses

Mean $\pm$ standard error of mean was used to summarize the data. The Student's *t* test was used to compare differences in means between two samples for normally distributed variables, with the Shapiro-Wilk normality test used to confirm normal distribution. For three or more groups, one-way ANOVA with Tukey's multiple comparison test (when comparing all groups) or Dunnett's test (when comparing test groups to one control group) or Sidak test (when comparing two select groups) were used, with $\alpha = 0.05$. The Kaplan-Meier method was used to generate median survival, and the hazard ratios estimated by log-rank test. Prism version 8.4.2 software (GraphPad) was used for statistical analysis and graph production.

SUPPLEMENTARY MATERIALS

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Fig. S1. α -V12 and α -V5 BsAb characteristics.

Fig. S2. TRBV and TRBC-specific BsAb treatment of normal human T cells in vitro.

Fig. S3. TRBC1, TRBV5-5 or TRBV12 engagement activates T cells against NALM6 B cells.

Fig. S4. α -C1 BsAb kills both TRBC1+ and TRBC2+ expressing T cells.

Fig. S5. Cell-surface CD3 and TRBV expression in T-cell cancer cell lines.

Fig. S6. TRBV specific BsAbs activate healthy T cells to kill T-cell cancer cells in vitro.

Fig. S7. TCR β sequencing to assess α -V12 and α -V5 and targeting specificity.

Fig. S8. TRBV5 family sequence alignment and structural analysis.

Fig. S9. TRBV-specific BsAbs activate human T cells to specifically kill T-cell cancers in vivo at low E:T ratio.

Table S1. Affinities and sequences of α -TRBV5-5, α -TRBV12, α -TRBC1, α -CD19 and α -CD3 single-chain variable fragments (scFvs).

Movie S1. α -V12 treatment protects NSG mice from Jurkat T cell xenograft induced hind-leg paralysis.

Data file S1. Raw data for main text and supplementary figures.

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Fig. 1. TRBV-specific BsAbs deplete cognate TRBV-expressing T cells while preserving the majority of non-targeted T cells. (A) Illustration depicting the proposed selective TRBV depletion strategy: Human T cells comprises 30 TRBV families, including TRBV1 (orange), TRBV5 (red), TRBV12 (cyan), TRBV20 (yellow), and TRBV30 (purple) expressing cells. α -V12 binds TRBV12 expressing T cells, leading to selective killing of the TRBV12 population while sparing the majority of the remaining non-TRBV12 T cells. (B) α -V5, α -V12 and α -C1 BsAbs are composed of α -CD3 scFv (orange) linked with α -TRBV5-5 (red), α -TRBV12 (cyan) and α -TRBC1 (grey) scFvs, respectively. Each scFv is composed of a variable heavy (V_H) and variable light (V_L) chain. (C) 1×10^6 normal human T cells were incubated with α -C1, α -V5 or α -V12 BsAbs (0.5 ng/ml) for 17 hours, followed by counting the number of surviving T cells and flow cytometric assessment of the TRBC and TRBV distribution in surviving T cells. Data are shown as the mean viable cell count from 5 different normal individuals. (D, E) Flow cytometry plots showing of percent of surviving T cells from 5 different normal human T cell donors after α -V5 BsAb or α -V12 BsAb (D) or α -C1 BsAb (E) treatment. In (C), (D) and (E), number of human replicates, $n = 5$. Number of repeated experiments, $N = 2$.

Fig. 2. TRBC1, TRBV5-5 or TRBV12 engagement activates T cells. (A) Illustration depicting bidirectional T-cell killing by α -C1, α -V5 and α -V12 BsAb. The conventional mechanism of action of α -C1, α -V5 and α -V12 involves crosslinking the T-cell activating CD3 molecule (using α -CD3 scFv) on T cell #1 with TRBC1, TRBV5-5, or TRBV12 (using anti-TRBC1, anti-TRBV5-5, or anti-TRBV12 scFvs, collectively shown as “ α -TRB”) on T cell #2, causing T cell #1 mediated killing of cell #2 (“i”). When target cell (cell #2) is also a T cell and can be activated by crosslinking with α -C1, α -V5 or α -V12, it may be able to function as an “effector” T cell and kill T cell #1 (“ii”). (B) Cartoons of α -CD3-CD19, α -C1-CD19, α -V5-CD19 and α -V12-CD19 BsAbs, composed of anti-CD19 scFv (black) linked to anti-CD3 (orange), anti-TRBC1 (grey), anti-TRBV5-5 (red), and anti-TRBV12 (cyan) scFvs. (C) and (D) 5×10^4 normal human T cells were incubated with 5×10^4 wild-type (WT) or CD19 knock-out (CD19-KO) NALM6 B cells (expressing luciferase) with the indicated BsAbs (0.5 ng/ml) for 17 hours. IFN γ ELISA was used to assess normal human T cell cytokine release (C) and luminescence was used to assess viable NALM6 B cells (D). (E, F) 5×10^4 target NALM6 B cells (expressing luciferase) were incubated with 5×10^4 normal human T cells, or TRBV5- and TRBV12-depleted T cells along with indicated BsAbs (0.5 ng/ml) for 17 hours. IFN γ detection was used to assess normal human T cell cytokine release (E) and luminescence was used to assess viable NALM6 B cells (F). (G, H) 5×10^4 target NALM6 B cells (expressing luciferase) were incubated with TRBV5 (“TRBV5 $^{+}$ ”)–enriched, or TRBV12 (“TRBV12 $^{+}$ ”)–enriched T cells, along with indicated BsAbs (0.5 ng/ml) for 17 hours. IFN γ detection was used to assess normal human T cell cytokine release (G) and luminescence was used to assess viable NALM6 B cells (H). In (C) to (H), bars represent mean $\pm$ standard error of mean using three different normal human T cell donors, $n = 3$. Number of repeated experiments, $N = 2$. In (C) and (D), *** $P < 0.001$, **** $P < 0.0001$, by one-way ANOVA with Dunnett’s multiple comparison test. In (E) and (F) * $P \leq 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not significant, by two-tailed paired t test. (G) and (H) **** $P < 0.0001$, by one-way ANOVA with Sidak multiple comparison test.

Fig. 4. TRBV-specific BsAbs kill T-cell cancer cells in vitro. (A) and (B) 5×10^4 normal human T cells were incubated with 5×10^4 Jurkat cells (A) or HPB-ALL cells (B) in the presence of the indicated concentrations of α -V12 (A) α -V5 (B) for 17 hours. The Jurkat and HPB-ALL cells expressed luciferase. Luminescence was used to assess viable Jurkat and HPB-ALL cells. The EC_{50} (M) for each BsAb is indicated in the corresponding graphs. (C) 5×10^4 normal human T cells were incubated with 5×10^4 wild-type (WT) or TCR gene-disrupted (TCR-KO) Jurkat cells in the presence of the indicated BsAbs (0.5 ng/ml) for 17 hours. (D) shows the aggregate data of percentage of tumor cells in each treatment condition using T cells from 3 different human donors (E) 5×10^4 normal human T cells were incubated with 5×10^4 wild-type (WT) or TCR gene-disrupted (TCR-KO) HPB-ALL cells in the presence of the indicated BsAbs (0.5 ng/ml) for 17 hours. All Jurkat and HPB-ALL cells expressed GFP. Flow cytometry was then used to assess CD3 and GFP expression. (F) shows the aggregate data of percentage of tumor cells in each treatment condition using T cells from 3 different human donors. In (C) and (E), the numbers beside density plots indicate the percentage of surviving cells. In (A), (B), (D) and (F), data represent mean $\pm$ standard error of mean using three different normal human T cell donors $n = 3$. Number of repeated experiments, $N = 2$. **** $P < 0.0001$. ns, not significant, by ANOVA with Sidak multiple comparison test.

Fig. 5. TRBV-specific BsAb kills patient-derived T-ALL cells in vitro. (A) Flow cytometric analysis of two T-ALL patient samples with circulating lymphoblasts expressing TRBV12. Numbers adjacent to the plots indicate the percentage of CD3⁺ cells that express TRBV12. (B) 5×10^4 normal human T cells were co-cultured with 5×10^4 patient derived T-ALL target cells (from patient 1 and patient 2) in the presence of the indicated BsAbs (0.5 ng/ml) for 17 hours. T cell cytokine release was assessed by measurement of IFN γ in the supernatant. (C) 5×10^4 normal human T cells were co-cultured with 5×10^4 patient derived T-ALL target cells (from patient 1) in the presence of the indicated BsAbs (0.5 ng/ml) for 17 hours. T cell activation and exhaustion markers were assessed by flow cytometry. In (B) and (C), bars represent mean $\pm$ standard error of mean from three technical replicates $n = 3$. (D) Flow cytometry histogram of HLA-A3 stained normal human T cells and patient derived T-ALL malignant cells. (E) and (F) 5×10^4 normal human T cells were co-cultured with 5×10^4 patient-derived T-ALL target cells in the presence of α -CD19 or α -V12 BsAbs (0.5 ng/ml) for 17 hours. Flow cytometric analysis of HLA-A3 and CD3 was then performed. Numbers adjacent to the plots indicate the numbers of cells counted by flow cytometry in a representative experiment (E), with data from 3 technical replicates, $n = 3$, shown in (F). **** $P < 0.001$ by one-way ANOVA with Sidak's multiple comparison test.

Fig. 6. TRBV-specific BsAbs specifically kill cancer cells in vivo. (A) Timeline of in vivo tumor experiments. (B) and (C) NSG mice were intravenously injected with 5×10^6 normal human T cells and 5×10^6 WT or TCR-KO Jurkat cells (B), or WT or TCR-KO HPB-ALL cells (C). All Jurkat and HPB-ALL cells expressed luciferase and GFP. Intraperitoneal pumps containing 100 μ g of α -CD19, α -V12 or α -V5 BsAb were placed in the animals four days after cell injection, and bioluminescence imaging (BLI) was performed on the indicated days. BLI data representative of one of two independent experiments with six NSG mice in each group are shown in (B) and (C). Number of animals in two experiments, $n = 5$, and $n = 6$. Number of independent experiments, $N = 2$. (D) Combined radiance value from two independent experiments with a total of eleven NSG mice in each group was measured on the indicated days. (E) and (F), circulating cancer cell and T cell counts were assessed from six different NSG mice for each cancer cell type. Flow cytometry on mouse blood collected on day 19 was used to detect circulating WT Jurkat or HPB-ALL cells ($CD3^+$, GFP⁺, top right quadrant), circulating TCR-KO Jurkat or HPB-ALL cells ($CD3^+$, GFP⁺, bottom right quadrant) or circulating normal human T cells ($CD3^+$, GFP⁺, top left quadrant) after the indicated treatments. In (E), flow cytometry data are representative of one of six NSG mice in each group. In (F), data combined from six NSG mice in each group are shown as mean $\pm$ standard error of mean. (G) and (H) Kaplan-Meier survival curves of WT or TCR-KO Jurkat (G) or HPB-ALL (H) bearing NSG mice after various treatments. Survival data were aggregated from two independent experiments with a total of eleven NSG mice in each group. Median overall survival is reported beside the survival curves. Jurkat WT/ α -CD19 versus Jurkat WT/ α -V12 hazard ratio (HR) = 0.18, **** $P < 0.0001$, log-rank (Mantel-Cox) test. HPB-ALL WT/ α -CD19 versus HPB-ALL WT/ α -V5, HR = 0.19, **** $P = 0.0001$, log-rank (Mantel-Cox) test. In (D) and (F), * $P \leq 0.05$, ** $P < 0.01$, **** $P < 0.0001$ by one-way ANOVA with Sidak's multiple comparison test.